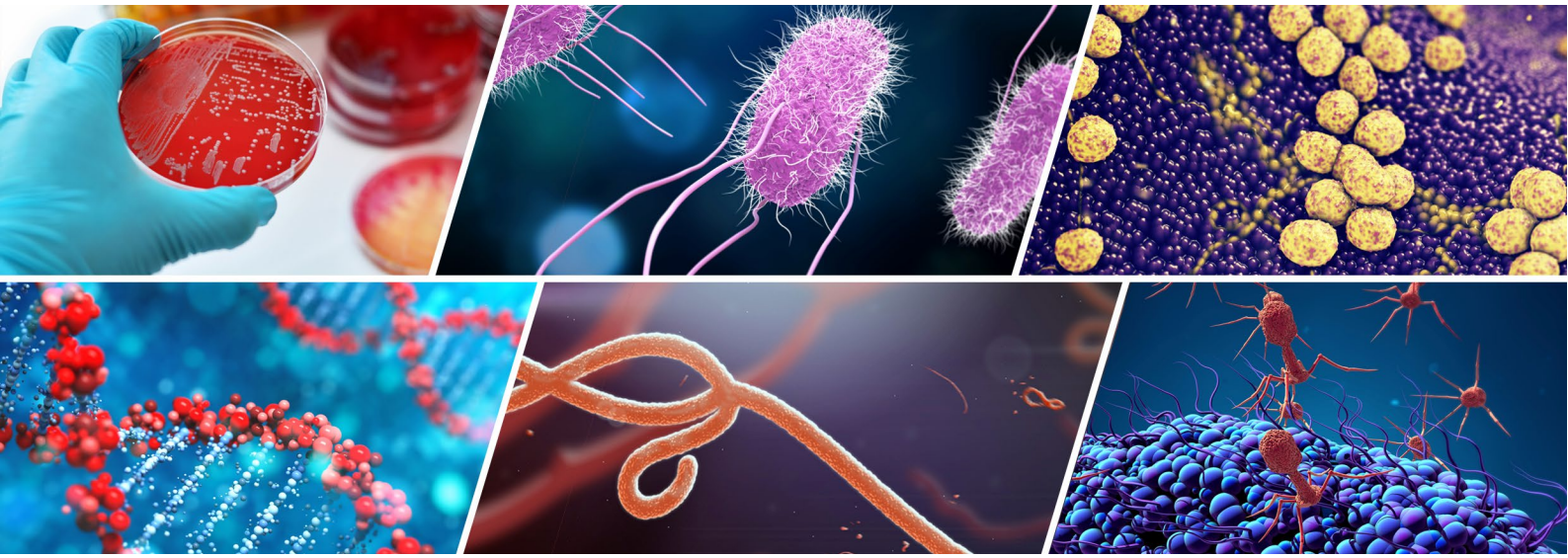
UNSW SYDNEY

SCHOOL OF BIOTECHNOLOGY AND BIOMOLECULAR SCIENCES



MICROBIOLOGY 1 (MICR2011)

SUBJECT GUIDE AND LABORATORY MANUAL

2022

Student Name
Student I.D.
Tutor

Table of contents

Course information 6
Staff contacts 6
Course outline 7
Graduate attributes developed in this course 8
Additional resources and support 8
Lecture program 9
Assessment 11
Adiministration matters 13
Academic honesty and plagarism 14

WEEK 1 RISK ASSESSMENT 17
WEEK 1 LABORATORY CLASS 18
1. Welcome! 18
1.1 Allocation of places 18
1.2 Introductions 18
1.3 Laboratory safety 18
2. Media for the cultivation of microbes 26
2.1 Understanding media recipe composition 26
2.2 Aseptic technique 26
2.3 Making and sterilising liquid broth and agar plates 28
3. MICR2011 course project 31

WEEK 2 RISK ASSESSMENT 35
WEEK 2 LABORATORY CLASS 36
1. DNA extraction of project samples for sequencing 36
2. Introduction to light microscopy 41
3. Staining bacteria for observation by light microscopy 46
4. Inoculating agar plates to obtain pure cultures and liquid broths as enrichment cultures 50

WEEK 3 RISK ASSESSMENT 55
WEEK 3 LABORATORY CLASS 56
1. Examination of cultures obtained from project samples 56
2. Selective and differential media 56
3. Biochemical tests used for phenotypic characterisation 62
4. Viable counts of microorganisms 69

WEEK 4 RISK ASSESSMENT 75
WEEK 4 LABORATORY CLASS 76
1. Winogradsky column 76
2. Soil based Microbial Fuel Cells 82
3. Maintenance of project microbes 84
4. High-throughput 16S (SSU) rRNA sequencing data 84
5. Viable counts of microorganisms (continued) 84

WEEK 5 RISK ASSESSMENT 87
WEEK 5 LABORATORY CLASS 88
1. Course project: sub-culturing, MALDI-TOF and storing project microbes 88
2. Introduction to sequence analysis 90
Exercise 1. Accessing software for phylogenetic analysis of SSU rRNA sequences 93

Exercise 2. Sequence browsing and alignment of SSU rRNA gene sequences 94
Exercise 3. Estimating evolutionary distance and creating phylogenetic trees 100
Exercise 4. BLAST search for closest relatives using the NCBI database.....102
3. Record the results of your Microbial Fuel Cell.....104

WEEK 7 RISK ASSESSMENT.....108
WEEK 7 LABORATORY CLASS.....109
1. DNA extraction, biochemical characterisation, and antibiotic resistance analysis from sub-cultured project isolates.....109
2. Assessment of DNA extract quantity and quality 110
3. PCR amplification and sequencing of SSU rRNA genes.....112
4. Record the results of your Microbial Fuel Cell.....113

WEEK 8 RISK ASSESSMENT.....117
WEEK 8 LABORATORY CLASS.....118

WEEK 9 RISK ASSESSMENT.....112
WEEK 9 LABORATORY CLASS.....113
1. Bacteriophage propagation and enumeration 113
2. Gene transfer by transduction.....115

WEEK 10 RISK ASSSSMENT 120
WEEK 10 LABORATORY CLASS.....121
1. Background.....121
2. Epidemiological investigation 125
3. ELISA analysis of mock serum samples 127
4. Tasks for the investigation team 129
5. Record results for PRD-1 phage enumeration and P1*kc* transduction experiments 130

Course Information	
Course Code	**MICR2011**
Course Name	**Microbiology 1**
Academic Unit	**School of Biotechnology and Biomolecular Sciences**
Level of Course	**Second year undergraduate**
Unit of credit	**6 UoC**
Session Offered	**T1**
Prerequisites	**BABS1201**
Hours per Week	**7 hours per week**
Number of Weeks	**10**
Commencement Date	**Week 1, Term 1, 2022**
Teaching time and locations	**http://timetable.unsw.edu.au/2022/MICR2011.html**

Staff				
Position	**Name**	**Email**	**Locations**	**Consultation times**
Course Convenor	**Associate Prof Jai Tree** Dr Gee Chong Ling	**j.tree@unsw.edu.au** g.ling@unsw.edu.au	**Rm3113, BioSc South E26** Rm220C, BioSc North D26	**By appointment**
Course Admin	**Ms Julna Zhao**	**j.zhao@unsw.edu.au**		**Email**
Technical Support	**Ms Tanjina Haque** Ms Agnee Amarnath	**tanjina.haque@unsw.edu.au** a.amarnath@unsw.edu.au	**BioSc Lab 11 & 12**	**In-class only**

NB: Successful completion of MICR2011 relied upon active participation in the laboratory component, reflecting by the attendance requirement and completion of assessment activities related to the course project. Should you have any question regarding your attendance of the laboratory activity, please contact the course convenor – Jai and Gee (j.tree@unsw.edu and g.ling@unsw.edu.au) to discuss your situation.

You MUST NOT assume alternative teaching is available at any random time during the term, alternative arrangement is subject to approval by the teaching team with the necessary supporting document provided, on a case-by-case basis.

Course Outline	
Course Description	This course is both for students majoring in microbiology and also those who wish to develop their knowledge and skills in microbiology. The course covers Introductory Microbiology including cell structure, function, physiology, and diversity. The course then focuses on specific areas of microbiology: Environmental Microbiology, Food Microbiology, Medical Microbiology, Microbes in Biotechnology, Virology, and Eukaryotic Microbiology.
Course Aims	Microbiology 1 aims to introduce you to microbes, their process, interactions and the techniques scientists use to study them.
Student Learning Outcomes	By the completion of this course students should know: - the characteristics of Bacteria, Eukarya, Archaea and Viruses; - the fundamental processes carried out by microbes; - the types of interactions that occur between microbial populations; - how microbial growth can be controlled - standard microbiological laboratory techniques and safe, efficient work practices; - how to conduct effective literature and experimental research; - how to communicate clearly and work constructively as a team.
Teaching Strategies	Lectures are used to introduce the concepts of microbiology and laboratory sessions are used to both complement the lecture material and provide practice in standard microbiological techniques. Laboratories sessions encourage teamwork. Online tutorials are additionally designed to support concepts presented in lectures and practiced in the laboratory, and support students in their research projects. The laboratory research project forms an essential element of the students' scientific training. The research project, as integrated with the other components of the course, have been designed in accordance with the UNSW Guidelines on Learning that Inform Teaching to: - teach students the process of scientific inquiry through progressive cycles of critical analysis of their research and their own thinking; - facilitate multidisciplinary thinking to reflect current research and professional practice in the sciences; - reinforce deep learning and promote collaborative inquiry; - integrate students' disciplinary understanding and research practice with the development of their communication skills, teamwork, and information literacy skills.
Major Topics (Syllabus Outline)	○ Microbial Cell Structure and Function ○ Evolution of Microbes and Microbial Diversity ○ Introduction to Archaea ○ Environmental Microbiology ○ Microbial Processes and Interactions ○ Medical Microbiology ○ Food Microbiology ○ Virology ○ Microbes in Biotechnology and Synthetic Biology ○ Eukaryotic Microbiology

Graduate Attributes Developed in this Course		
Science Graduate Attributes	Degree of development *0 = no development* *1 = minimal* *2 = minor* *3 = major*	Activities / Assessment
Research, inquiry and analytical thinking abilities	3	Guided laboratory practicals, independent and collaborative lab research, assessment of open-ended investigations.
Capability and motivation for intellectual development	3	Semester project and associated report; concept quizzes to review procedural and applied thinking.
Ethical, social and professional understanding	2	Lectures address ethical and social issues relevant to the field of microbiology; lectures and discussions on current research in microbiology.
Communication	3	Writing and feedback on Semester Project Report Guidance in the development of scientific writing skills using tutorial materials.
Teamwork, collaborative and management skills	3	Collaborative lab research projects; facilitation of group discussions in Moodle; guided peer review of written research reports.
Information literacy	3	Guided research writing tutorial and critical literacy assessments; scientific literacy tutorial.

Additional Resources and Support	
Text Books	Brocks Biology of Microorganisms. 15th edition Pearson Or Prescott, Harley and Klein. Microbiology 10th ed., McGraw Hill. NOTE: Earlier editions are satisfactory; however specific references to page numbers may vary.
Laboratory Manual	A course laboratory manual is required and students may access an eCOPY through Class Notebook (MS Teams) and also PDF-copy of the Manual through the MICR2011 Moodle page.
Internet Site	Students enrolled in the course have access to the MICR2011 Moodle website and are required to access this regularly. Announcements will be made through Moodle email system. Links to online tutorial material will be provided on Moodle.
Equipment Required	A lab coat, covered shoes, and safety glasses must be worn in all laboratory classes.

Course Schedule					
Week	**Date**	**Lecture**		**Practical**	**Tutorial**
Week 1	Feb 14th	Introduction to MICR2011	JT	Aseptic technique	Sterilisation
	Feb 17th	Unicellular structure and function	JT		
	Feb 18th	Microbial phylogeny	JT		
Week 2	Feb 21st	Course Project	GL	Project DNA extraction	Phylogeny and classification
	Feb 24th	Microbial bioinformatics	MT		
	Feb 25th	Microbial metabolism 1	BB		
Week 3	Feb 28th	Microbial metabolism 2	BB	Biochemical techniques for characterisation	Biochemical techniques for characterisation
	Mar 3rd	Microbial evolution	JT		
	Mar 4th	Environmental microbiology 1	BF		
Week 4	Mar 7th	Environmental microbiology 2	BF	Environmental Microbiology (MFCs & Winogradsky column)	Environmental Microbiology (The Winogradsky column)
	Mar 10th	Microbial interactions	SE		
	Mar 11th	Archaea	GL		
Week 5	Mar 14th	Bacterial Disease	JT	16S rDNA sequence analysis	Project report writing
	Mar 17th	Introduction to Immunology	NW		
	Mar 18th	MID-TERM EXAM			
Week 6	FLEX WEEK				
Week 7	Mar 28th	The Human Microbiome	NCR	Sanger sequencing project microbes	Antibiotics
	Mar 31st	Foodborne pathogens	PW		
	April 1st	Antibiotics and antibiotic resistance	JT		
Week 8	Apr 4th	Introduction to viruses	PW	Food Microbiology	Viruses
	Apr 7th	HIV and Hepatitis C	PW		
	Apr 8th	Bacteriophages	PW		
Week 9	Apr 11th	Fungi 1	ML	Bacteriophages	Fungi
	Apr 14th	Fungi 2	ML		
	Apr 15th	GOOD FRIDAY			
Week 10	Apr 18th	EASTER MONDAY		Ebola outbreak!	
	Apr 21st	Microbes & Synthetic Biology 1	MB		
	Apr 22nd	Microbes & Synthetic Biology 2	DG		
	Apr 22nd	Course Summary	JT		

Lecturers:

A/Prof Jai Tree (JT)
A/Prof Belinda Ferrari (BF)
Dr Nancy Wang (NW)
Dr Megan Lenardon (ML)
Prof Mark Tanaka (MT)
A/Prof Suhelen Egan (SE)
Prof Peter White (PW)
Dr Matt Baker (MB)
A/Prof Brendan Burns (BB)
Dr Gee Chong Ling (GL)
Dr Natalia Castano Rodriguez (NCR)
Dr Dominic Glover (DG)

Laboratory classes are held in Biosciences South (Building E26) laboratories 11 and 12 on Level 1. You are required to bring a laboratory coat and closed shoes.

Online tutorials are accessed through the MICR2011 Moodle website.

NB: For each laboratory you must complete the Pre-Lab Quiz before the lab session. The Pre-Lab Quiz can be accessed from the MICR2011 Moodle page.
For student attending the course in ONLINE mode, you will need to complete Pre-Lab worksheet (equivalent to Pre-Lab Quiz) before the online lab session.

Assessment			
Assessment type	**Mark**	**Description**	**Due date**
Mid-session exam	20%	Examines material covered in lectures, practicals, and tutorials up to and including Week 4 of the course.	18th March
Online tutorials	16%	Completion of ALL online tutorials (8x).	Each week
Course project report	30%	Report on microbe isolated and identified during the course project *-500 words rationale due Week 3 (5%)* *-Draft report due Week 7 (5%)* *-Peer review of draft due Week 8 (5%)* *-Final report due Week 10* (15%)	Week 3-10
Final theory examination	30%	Examines material covered in all lectures, practicals, and tutorials for entire course.	TBA
Pre-lab quiz (Pre-lab worksheet – ONLINE)	4%	Pre-lab quiz must be completed to participate in the practical class each week.	Each week
Weekly revision quiz	No marks assigned	Revision quiz for weekly course content	Each week

Further information

UNSW grading system: https://student.unsw.edu.au/grades
UNSW assessment policy: https://student.unsw.edu.au/assessment

Assessment criteria and standards

Detailed assessment criteria together with grading rubric will be provided through Moodle site

Assessment structure of Course Project Report:

Course Project – 30%

Assessment	Grade	Submission method	Submission Deadline	Feedback/Marking	Portal
Rationale – ~500 words	5%	Group	Week 3 Friday 1700	Week 4 Friday 1700	Turnitin
Draft^ - ~1500 words	5%	Group	Week 7 Friday 1700	Week 9 Monday	Workshop Tool
Peer Review	5%	Individual	Week 8 Friday 1700	Week 9 Monday	Workshop Tool
Final Report – ~3000 words -Reflective	15%	Individual	Week 10 Friday 1700	Week 12 Friday 1700	Turnitin
Student Contribution Form*	-	Individual	After Rational, Draft and Final Report		MS Form

**Student Contribution Form must be submitted for any review of group marks, group mark may be reviewed and adjusted according to the contribution data provided by the individual members of the group*

*^Each student will be peer reviewing **THREE** other submissions*

Submission method

ALL assessment submission will be through Moodle, this include:
-Moodle Quiz – Mid-session and Final theory exam
-Moodle H5P Lessons – Pre-Lab quiz and Tutorial Quiz
-Turnitin – Rationale and Final report (CPR)
-Moodle Workshop – Draft and Peer Review (CPR)

Feedback on assessment

-Feedback to be provided through Moodle with rubric comment on ***individual*** basis – Mid-session exam, online tutorials, pre-lab quizzes, peer review activity and final report.
-Feedback to be provided through Moodle with rubric comment from demonstrator on project ***group*** – rationale and draft.

Administration Matters	
Expectations of Students	A pass in MICR2011 is conditional upon a satisfactory performance in the practical program. A satisfactory performance means that you have: - attended at least 80% of the practical classes (an attendance record is kept), and; - kept an accurate and up-to-date laboratory manual, including the recording of all data and completion of calculations and questions.
Assignment Submissions	Requirements vary with each assigned task. Your tutor will advise accordingly.
Workplace Health and Safety[1]	Information on relevant Workplace Health and Safety policies and expectations at UNSW: https://safety.unsw.edu.au.
Assessment Procedures	Students who believe that their performance, either during the session or in the end of session exams, may have been affected by illness or other circumstances may apply for special consideration. Applications can be made for compulsory class absences such as (laboratories and tutorials), in-session assessments tasks, and final examinations. Students must make a formal application for Special Consideration for the course/s affected as soon as practicable after the problem occurs and within three working days of the assessment to which it refers. Students should consult the A-Z section of the Student Guide, particularly the section on "Special Consideration", for further information about general rules covering examinations, assessment, special consideration and other related matters. This is information is published free in your UNSW Student Diary and is also available on the web at: https://student.unsw.edu.au/guide HOW TO APPLY FOR SPECIAL CONSIDERATION Applications must be made via Online Services in myUNSW. You must obtain and attach Third Party documentation before submitting the application. Failure to do so will result in the application being rejected. Log into myUNSW and go to My Student Profile tab > My Student Services channel > Online Services > Special Consideration. After applying online, students must also verify supporting their documentation by submitting to UNSW Student Central: • Originals or certified copies of your supporting documentation (Student Central can certify your original documents), and • A completed Professional Authority form. The supporting documentation must be submitted to Student Central for verification within three working days of the assessment or the period covered by the supporting documentation. Applications which are not verified will be rejected. Students will be contacted via the online special consideration system as to the outcome of their application. Students will be notified via *their official university email once an outcome has been recorded.*

	SUPPLEMENTARY EXAMINATIONS: The University does not give deferred examinations. However, further assessment exams may be given to those students who were absent from the final exams through illness or misadventure. Special Consideration applications for final examinations and in-session tests will only be considered after the final examination period when lists of students sitting supplementary exams/tests for each course are determined at School Assessment Review Group Meetings. Students will be notified via the online special consideration system as to the outcome of their application. It is the responsibility of all students to regularly consult their official student email accounts and myUNSW in order to ascertain whether or not they have been granted further assessment. All supplementary exams will be held during the week Monday 23 May – Friday 27 May May, 2022.
Equity and Diversity	Those students who have a disability that requires some adjustment in their teaching or learning environment are encouraged to discuss their study needs with the course Convenor prior to, or at the commencement of, their course, or with the Equity Officer (Disability) in the Equity and Diversity Unit (9385 4734 or http://www.studentequity.unsw.edu.au/) Issues to be discussed may include access to materials, signers or note-takers, the provision of services and additional exam and assessment arrangements. Early notification is essential to enable any necessary adjustments to be made. Information on designing courses and course outlines that take into account the needs of students with disabilities can be found at: https://www.unsw.edu.au/sites/default/files/uploads/Accessibility_Guidelines.pdf

Grievance Policy	**School Contact**	**Faculty Contact**	**University Contact**
	Biosciences Student Office Student Advisor BABStudent@unsw.edu.au Student Grievance Officer Megan Lenardon m.lenardon@unsw.edu.au	Dr Gavin Edwards g.edwards@unsw.edu.au Tel: 9385 4652	University Counselling Tel: 9385 5418 https://student.unsw.edu.au/complaint

Academic integrity, referencing and plagiarism

Referencing is a way of acknowledging the sources of information that you use to research your assignments. You need to provide a reference whenever you draw on someone else's words, ideas or research. Not referencing other people's work can constitute plagiarism.
Further information about referencing styles can be located at https://student.unsw.edu.au/referencing

Academic integrity is fundamental to success at university. Academic integrity can be defined as a commitment to six fundamental values in academic pursuits**:** honesty, trust, fairness, respect, responsibility and courage.[2] At UNSW, this means that your work must be your own, and others' ideas should be appropriately acknowledged. If you don't follow these rules, plagiarism may be detected in your work.
Further information about academic integrity and **plagiarism** can be located at:

- The *Current Students* site https://student.unsw.edu.au/plagiarism**,** and
- The *ELISE* training site http://subjectguides.library.unsw.edu.au/elise/presenting

The *Conduct and Integrity Unit* provides further resources to assist you to understand your conduct obligations as a student: https://student.unsw.edu.au/conduct.

Plagiarism is the presentation of the thoughts or work of another as one's own.
Examples include:

- Direct duplication of the thoughts or work of another, including by copying work, or knowingly permitting it to be copied. This includes copying material, ideas or concepts from a book, article, report or other written document (whether published or unpublished), composition, artwork, design, drawing, circuitry, computer program or software, web site, Internet, other electronic resource, or another person's assignment without appropriate acknowledgement.
- Paraphrasing another person's work with very minor changes keeping the meaning, form and/or progression of ideas of the original.
- Piecing together sections of the work of others into a new whole.
- Presenting an assessment item as independent work when it has been produced in whole or part in collusion with other people, for example, another student or a tutor.
- Claiming credit for a proportion a work contributed to a group assessment item that is greater than that actually contributed.
- Submitting an assessment item that has already been submitted for academic credit elsewhere may also be considered plagiarism.
- The inclusion of the thoughts or work of another with attribution appropriate to the academic discipline does *not* amount to plagiarism.

Students are reminded of their Rights and Responsibilities in respect of plagiarism, as set out in the University Undergraduate and Postgraduate Handbooks, and are encouraged to seek advice from academic staff whenever necessary to ensure they avoid plagiarism in all its forms.
The Learning Centre website is the central University online resource for staff and student information on plagiarism and academic honesty. It can be located at: https://student.unsw.edu.au/plagiarism

The Learning Centre also provides substantial educational written materials, workshops, and tutorials to aid students, for example, in:

- Correct referencing practices.
- Paraphrasing, summarising, essay writing, and time management.
- Appropriate use of, and attribution for, a range of materials including text, images, formulae and concepts.

Students are also reminded that careful time management is an important part of study and one of the identified causes of plagiarism is poor time management. Students should allow sufficient time for research, drafting, and the proper referencing of sources in preparing all assessment items.

[2] International Center for Academic Integrity, 'The Fundamental Values of Academic Integrity', T. Fishman (ed), Clemson University, 2013.

Additional support for students
• The Current Students Gateway: https://student.unsw.edu.au/ • Academic Skills and Support: https://student.unsw.edu.au/academic-skills • Student Wellbeing, Health and Safety: https://student.unsw.edu.au/wellbeing • Disability Support Services: https://student.unsw.edu.au/disability-services • UNSW IT Service Centre: https://www.it.unsw.edu.au/students/index.html

WEEK 1
LABORATORY

OVERVIEW

This week we welcome you to the BABS teaching laboratories in the Biosciences South Building (E26). The laboratories you will be studying in are Labs 11 and 12 on level 1 of the building. In this three-hour lab class, we will be focussing on laboratory safety, making media for the cultivation of microorganisms, and choosing an environment to sample so that you can isolate a microorganism for your course project.

LEARNING OBJECTIVES

- Understand the risks involved in working with microorganisms in a PC2 facility and the personal protective equipment and behaviors required to manage those risks
- Understand how microorganisms are cultivated in the laboratory
- Begin to develop skills necessary to isolate and purify microorganisms

ACTIVITIES

1. Welcome!
   a. Allocation of places
   b. Introductions
   c. Laboratory safety

2. Media for cultivation of microbes:
   a. Understanding media recipe composition (energy, elements and vitamins)
   b. Making and sterilising liquid broth and agar plates
   c. Aseptic technique and labelling

3. Introduction to MICR2011 course project

Contents:

WEEK 1 RISK ASSESSMENT 17
WEEK 1 LABORATORY CLASS 18
1. Welcome! 18
1.1 Allocation of places 18
1.2 Introductions 18
1.3 Laboratory safety 18
Microbiology Laboratory Safety Rules 19
Risk management 20
Risk Management Form 22
2. Media for the cultivation of microbes 26
2.1 Understanding media recipe composition 26
2.2 Aseptic technique 26
2.3 Making and sterilising liquid broth and agar plates 28
i) Luria broth 28
Materials 28
Procedure 28
ii) Pouring Luria agar plates 29
Materials 29
Procedure 29
3. MICR2011 course project 31

WEEK 1 RISK ASSESSMENT

BABS Teaching Laboratory	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk2
Student Risk Assessment		

Hazards	Risks	Controls
General	See below	**<u>At all times</u>** follow demonstrators instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Collecting biological /environmental samples	Inhalation of bio-aerosol particles (microorganisms) leading to inflammation, allergy, or to serious systemic infection by secondary pathogens.	PPE (lab coats, closed in shoes & gloves as required). Work in PC2 laboratory or Biological Safety Cabinet (BSC) if required e.g. for respiratory pathogens.
Sharps	Sharp wire injury	Take care when handling wire and place any unused sharp items in the sharps containers provided.
Handling hot items	Electric shock/ electrocution burns	Ensure you are using heat protective mitts when handling hot objects. Stand to side of the water bath when opening it to allow steam to dissipate before removing items. Avoid water/spillages when working with electrical items.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 1 LABORATORY CLASS

1. Welcome!

1.1 Allocation of places

Students should proceed to the appropriate laboratory (Lab 11 or 12 in Biosciences South E26 as advised) where practical class groups will be organised.

1.2 Introductions

Students and tutors should get acquainted:

- Briefly introduce individual backgrounds.
- A brief discussion of course objectives and requirements.
- Explanation of assessable components of this course.
- Brief overview of laboratory and tutorial programs.

1.3 Laboratory safety

Note: In addition to reading and signing off on the material below all students will be required to complete the laboratory BABS Health and Safety Quiz in Moodle before next week's class (Week 2). You will not be able to proceed in further practical classes until you have completed this quiz.

In the laboratory, individuals are exposed to hazards not normally found in a regular classroom. It is essential that students follow all laboratory rules and regulations. Failure to follow established rules may result in dismissal of the individual from the class. Laboratories have certain standard safety equipment. These typically include:

- general-purpose fire extinguisher
- fire blanket
- eyewash
- safety shower
- cut-off switches for electrical and gas outlets.

It is the responsibility of the student to locate and know how to use the general safety equipment in the laboratory. Additionally, students should be aware of exits from the room in case of emergency, how to summon Campus Security (9385 6666), and how to obtain emergency medical assistance.

The microbiology lab has some additional safety considerations. Since individuals work with potentially pathogenic organisms care must be taken to prevent possible infection or transmission of the organisms from the laboratory. Students must wear protective clothing (including lab coats, safety glasses and closed in shoes) while working in the laboratory. Lab coats may not be worn outside the laboratory. Gloves will be provided and students must wear gloves when handling chemicals or pathogenic microorganisms.

Aseptic technique must be followed while working with microorganisms, and appropriate instruction in aseptic technique will be provided. Bench-tops must be disinfected after lab classes using the disinfectant provided.

You MUST wash your hands before leaving the lab.

Microbiology Laboratory Safety Rules

All materials and clothes other than those needed for the laboratory are to be kept away from the work area in the lockers provided.

- A lab coat or other protective clothing must be worn during the lab. Lab clothing is not to be worn outside of the laboratory.
- Items heavily contaminated with bacteria must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container.
- Aseptic technique must be observed at all times. No eating, drinking, application of cosmetics or smoking is allowed. Mouth pipetting is not allowed.
- Cuts and scratches must be covered with appropriate dressing.
- Long hair should be tied back while in the lab.
- All accidents, cuts, and any damaged glassware or equipment should be reported to the lab demonstrator immediately.
- Sterilization techniques will involve the use of Bunsen burners, which constitute a fire and burn hazard. Keep all combustibles away from the Bunsen burners.
- It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc.)
- Cultures may not be removed from the lab.
- Visitors are not allowed in the lab.

Health and Safety Precautions for Electronic devices including laptop computers and mobile phones:

- Mobile phones: For your own safety please ensure your mobile phone is placed in a plastic zip lock bag.
- Computers and tablets: Please cut a section of benchcoat (this will be provided in the lab class) and place your device on this on the lab bench to separate it from your other laboratory work. DO NOT wear gloves when using these devices.

Laboratory clean up instructions

At the end of each practical class you must clear your bench space. This includes:

1) Discard liquid(biological) waste into the aqueous waste container located at the sink.
2) Discard solid waste (e.g. agar plate) into the Biological waste bin located near the end of the bench.
3) Discard sharps (e.g. pipette tips, slides & coverslips) into the sharps container located on the bench. Do not leave Gram stained slides unattended at the staining station.
4) Return used practical materials to the tutor station.
5) Store practical materials accordingly (refer to your demonstrator's instruction).
6) Placed empty tip boxes and microfuge tube jars in the box located at the back of the lab.

I have read and understand the above rules and agree to follow them.

Signed_____Date_____

Name _____

Risk management:

For every task we perform in the laboratory that involves new equipment or substances that are potentially hazardous we are legally required to prepare a risk management plan (Workplace Health and Safety Act and Regulation 2011).

What is Risk Management?

It is about identifying hazards, assessing them and controlling the risk of the hazards. When identifying hazards consider where, how and who is carrying out work. The level of risk is assessed and used to prioritise implementation of risk control measures.

The risk control measures are:

- Eliminate the hazard(s)
- Substitute for something less hazardous
- Isolate the hazard from people
- Engineering controls e.g. plant guarding, work in fume cupboard or biosafety cabinet
- Administrative controls e.g. training, safe work procedures, signage
- Personal protective equipment (PPE) e.g. lab coat, safety glasses, gloves

To prepare a risk management plan/assessment you need to do the following:

- Identify Hazards: Source of potential harm to people, property or environment
- Assess Risks: Chance of something happening - consequence vs likelihood
- Eliminate or Control Risks

For the practical based studies you will undertaking in this course you will be required to read and sign the risk management plan associated with each individual laboratory class to provide evidence you have understood the associated risks and the control measures that have been put in place to minimise these risks. The risk management plans have been prepared by experienced staff however to help you understand this process we will ask you to identify the hazards and risks you may encounter in a microbiology lab and control measures we can use to minimise these risks and then discuss this with your fellow students and demonstrator.

An example of a risk management form for working with a microscope is shown on the next page. Note all risk assessments at UNSW are now submitted on-line using the

SafeSys database (http://safety.unsw.edu.au/node/93/).

Risk Management Form

Document details			
Document Number	SCI-BABS-RMF-1603	Title	Use of the light Microscope
Author	Jani O'Rourke	Approver	Jani O'Rourke
Approval Status	Approved	Approval Date	02/06/2018
Faculty	Science	School	School of Biotechnology and Biomolecular Science
Next Review date	02/06/2021		

Risk Management Details	
Risk Management Form Description	Use of the light microscope for the visualisation of bacterial and fungal specimens
Locations	All BABS labs
Persons at Risk	Workers, Students, Visitors
Consultation Process	Persons must read this form
Related Legislation, Standards, Codes of Practice etc	WHS Act 2011; WHS Regulations 2011 AS/NZS 2243 Safety in Laboratories 2010 Appropriate UNSW Procedures and Guidelines

Hazards and Risks	
Hazard Task	Use of glass microscope slides and glassware
Hazard Category	Sharps/Needlesticks
Associated Harm	Cuts to hands and fingers from broken slides
Existing Controls	Sharp and glass bins are provided for immediate disposal of broken glass slides
Risk Consequence	Minor
Risk Likelihood	Unlikely
Risk Rating	Low
Additional Controls	-
Hazard Task	Use of electrical equipment
Hazard Category	Electrical
Associated Harm	Electrocution
Existing Controls	Annual tagging and testing. If a problem occurs staff are to switch off the machine, place an out of service notice on the equipment and notify the supervisor immediately. An electrical cut-off switch is located at the front tutor station.
Risk Consequence	Moderate
Risk Likelihood	Rare
Risk Rating	Medium
Additional Controls	-
Hazard Task	Sore back, neck or eyes

Hazards and Risks	
Hazard Category	Ergonomic
Associated Harm	Back, neck or eye strain due to extended use or too high intensity of light source
Existing Controls	4 hour maximum booking allowed. Adjustable chairs provided. Training and risk assessment
Risk Consequence	Minor
Risk Likelihood	Unlikely
Risk Rating	Low
Additional Controls	-
Hazard Task	Exposure to biological materials
Hazard Category	Biologicals
Associated Harm	Infection
Existing Controls	PPE (lab coats, gloves, closed-in shoes, tied back hair, safety glasses for handing specimens), appropriate disinfection process of specimens and any spills
Risk Consequence	Minor
Risk Likelihood	Rare
Risk Rating	Low
Additional Controls	-

Other Risk Management Details	
Date All Controls Implemented	02/06/2015
Emergency Procedures	Secure your work and turn off power sources and gas supply (if applicable), remove PPE. Advise others in the laboratory of the situation and report any fault or incident causing the shutdown to Lab Supervisor, School H&S representative or Facilities (FM Assist x55111). Fire: Cover the fire with a fire blanket if it can be fully contained underneath the blanket otherwise use a fire extinguisher. Carbon dioxide or dry powder is suitable. Be prepared for re ignition, especially if equipment is involved; do not leave the scene unless fumes or smoke become hazardous. Exposure; remove contaminated protective clothing and examine your clothes for contamination which may have soaked through. If day clothing is contaminated, remove, and wash skin under running water immediately for 15 minutes or until medical attention arrives. Assess if any person requires medical attention – contact first aid officers if required. Advise others of the situation and clear the laboratory if required. Spill: See SCI-BABS-SWP-665 for dealing with biological spills See SCI-BABS-SWP-666 for dealing with chemical spills. Report incident to Lab Supervisor and School H&S representative. For emergencies contact UNSW Security on x 56666.
Competency and Training Required	Training by experienced technical staff
Competency Levels	Read Document

Describe hazards and risks associated with working with unidentified microorganisms.

Describe control measures that can be put in place to mitigate these risks.

RISK RATING METHODOLOGY AND MATRIX

Consider the Consequences	Consider the Likelihood	Calculate the Risk
Consider: What type of harm could occur (minor, serious, death)? Is there anything that will influence the severity (e.g. proximity to hazard, person involved in task etc.). How many people are exposed to the hazard? Could one failure lead to other failures? Could a small event escalate?	Consider: How often is the task done? Has an accident happened before (here or at another workplace)? How long are people exposed? How effective are the control measures? Does the environment effect it (e.g. lighting/temperature/pace)? What are people's behaviours (e.g. stress, panic, deadlines) What people are exposed (e.g. disabled, young workers etc.)?	1.Take the consequences rating and select the correct column 2.Take the likelihood rating and select the correct row 3. Select the risk rating where the two ratings cross on the matrix below. **VH = Very high, H = High, M = Medium, L = Low**
5. Severe: death or permanent disability to one or more persons **4. Major**: hospital admission required **3. Moderate**: medical treatment required **2. Minor**: first aid required **1. Insignificant**: injuries not requiring first aid	**A. Almost certain**: expected to occur in most circumstances **B. Likely**: will probably occur in most circumstances **C. Possible**: might occur occasionally **D. Unlikely**: could happen at some time **E. Rare**: may happen only in exceptional circumstances	

		CONSEQUENCES				
		1	2	3	4	5
LIKELIHOOD	A	M	H	H	VH	VH
	B	M	M	H	H	VH
	C	L	M	H	H	VH
	D	L	L	M	M	H
	E	L	L	M	M	M

Risk level	Required action
Very high	**Act immediately**: The proposed task or process activity must not proceed. Steps must be taken to lower the risk level to as low as reasonably practicable using the hierarchy of risk controls
High	**Act today:** The proposed activity can only proceed, provided that: (i) the risk level has been reduced to as low as reasonably practicable using the hierarchy of risk controls and (ii) the risk controls must include those identified in legislation, Australian Standards, Codes of Practice etc. and (iii) the document has been reviewed and approved by the Supervisor and (iv) a Safe Working Procedure or Safe Work Method has been prepared and (v) the supervisor must review and document the effectiveness of the implemented risk controls
Medium	**Act this week**: The proposed task or process can proceed, provided that: (i) the risk level has been reduced to as low as reasonably practicable using the hierarchy of controls and (ii) the document has been reviewed and approved by the Supervisor and (iii) a Safe Working Procedure or Safe Work Method has been prepared.
Low	**Act this month:** Managed by local documented routine procedures which must include application of the hierarchy of controls.

2. Media for the cultivation of microbes

2.1 Understanding media recipe composition

Notwithstanding the contributions of Leeuwenhoek (first microscope observations), Jenner (first vaccine), Pasteur (vaccines and pasteurisation) and Lister (antiseptic surgery), it was not until Robert Koch's laboratory invented the petri dish and the agar plate that it was possible to isolate microorganisms. The isolation and cultivation of microorganisms allowed for the confirmation of germ theory and is fundamental to microbiology. Thus, it is important to understand how to handle and grow microbes. This practical class will introduce students to the composition of defined and complex media for growing microbes and techniques for media production.

While advances in molecular techniques have allowed for the study of microorganisms in their specific natural environments the selection and isolation of individual organisms is still an important and necessary tool for microbiologists. As such, culture media that allows for the cultivation of microorganisms in the laboratory is required. Culture media must provide an energy source (electron donor) and a range of macronutrients and micronutrients with the exact requirements and composition of these elements varying between individual microbial species. Macronutrients include carbon, hydrogen, oxygen, nitrogen, sulphur, phosphorus, potassium, calcium, magnesium and iron. Micronutrients or trace elements include manganese, zinc, copper, cobalt and molybdenum.

In addition, some organisms may require specific growth factors, as they are unable to synthesise certain organic compounds from the available nutrients. The need for growth factors is due to absent or dysfunctional metabolic pathways in these organisms. In a broad sense, there are three types of growth factors:

- Purines and pyrimidines: required for synthesis of nucleic acids (DNA and RNA),
- Amino acids: required for the synthesis of proteins,
- Vitamins: needed as coenzymes and functional groups of certain enzymes.

Culture media can be divided into two main types – defined and complex. In defined media all the chemical components of the media are known and quantified. It is often used in situations where it is important to know exactly what is being metabolised by the microorganism. By comparison, complex media will have one or more undefined components such as peptone, meat extract or yeast extract. Here we don't know the exact concentrations of all compounds in the media. Complex media is great when you don't know the specific nutritional requirements of an organism.

While both liquid and solid media are used in microbiology laboratories, the isolation of single colonies for the growth of pure cultures (one microbial lineage in isolation) is performed using solid media.

2.2 Aseptic technique

In order to identify and carry out detailed studies on microbes it is essential that a pure culture be used. Otherwise you can't unambiguously attribute a trait of interest to a specific organism. For example you can't attribute a disease to a specific organism if it is mixed with others. It has to show virulence on its own. The ability to obtain pure cultures and

maintain them free of external contamination is a basic microbiological technique. In this exercise you will have the opportunity of practicing sub-culturing and streak-plating techniques and of isolating organisms in pure culture. It all revolves around something we call aseptic technique. Accidental contamination may ruin your results completely.

Aseptic technique is largely a matter of common sense, but it is essential to realise that bacteria and fungi are present almost everywhere. Correct methods of handling cultures and apparatus must be perfected.

Consider carefully and remember the following points:

- Air contains many bacterial and fungal cells carried on dust particles or in water droplets.
- Any surface exposed to air quickly becomes contaminated, and if material is to be kept sterile exposure to air must be minimized.
- Instruments which can be sterilised by heating in a Bunsen flame (e.g. metal inoculating loops) can be left exposed, but they must be flamed thoroughly before use, and again before being replaced in the holder.
- Items of equipment that cannot be treated in this way are sterilised in wrappings or containers from which they must not be removed until actually needed. They must not be allowed to touch non-sterile surfaces during use. Plugs and caps of tubes and bottles must not be laid on the bench nor must sterile containers be left open to collect falling dust.
- Clothes, hair, skin and breath all carry a heavy microbial load and where strict asepsis (sterility) is essential (eg. surgery) sterilised gowns, caps, gloves and masks are worn. Even in normal microbiological work care must be taken to prevent contamination from the above-mentioned sources.
- A clean laboratory is the foundation of good aseptic technique.
- Microbial contamination in the lab is most often due to currents of non-sterile air. Ideally biosafety cabinets are used to minimize contamination. In the absence of a biosafety cabinet, a Bunsen burner can be used to create an updraft, thereby preventing contamination by settling dust particles. This protection can also be supplemented by keeping all windows and doors shut and by cutting down movement within the laboratory.
- Before commencing any operation, all necessary materials should be assembled on the bench for convenient access with provision for protecting sterile objects until needed.

At this stage of this practical class each tutor group will be divided into four mini-groups (with 4 students each). You will also use these mini-groups for the main course project**.**

Each group will initially be allocated to one of the following exercises:

1. Pouring agar plates,
2. Making Luria broths,
3. Or choosing your environment for the study of its indigenous microbes

Each group will then rotate through each exercise. It is anticipated that each exercise will take roughly half an hour.

2.3 Making and sterilising liquid broth and agar plates

Luria broth, originally know as Lysogeny broth, was devised by Giuseppe Bertani in 1952 for growing pathogenic *Shigella* lineages. It is now commonly used for the growth of enteric species such as the research workhorse *Escherichia coli* and in recombinant DNA work. It is a complex media that is easy to prepare and contains a wide range of nutrients with the following ingredients:

- Tryptone: peptides and peptones providing essential amino acids
- Yeast extract: vitamins, trace elements and certain organic compounds
- NaCl: sodium ions for transport and osmotic balance.

i) Luria broth

Materials:

- Tryptone
- Yeast extract
- Sodium chloride
- 50ml polyproylene centrifuge tube
- Spatula, weighing boat, balance
- Specimen container and pipettes.

30ml polycarbonate tube

Procedure:

1. Using a spatula, weighing boat and a balance, each student should weigh out the following ingredients into a conical (Erlenmeyer) flask. Rinse and dry the spatula between different chemicals to avoid mixing (no double dipping).
   - Tryptone: 0.2 g
   - Yeast extract: 0.15 g
   - NaCl: 0.2 g

2. Dissolve in 20 mL of distilled water.

3. Using a pipette aliquot 2 x 10 mL volumes into the 50 ml tube provided. Label the lid of your bottles with your name and date and place in the tray provided. The tubes will be autoclaved by the technical staff and returned to you for use in next week's class.

Please ensure lids are slightly loosened before handing them in. If they are tightly sealed the pressure generated during autoclaving may cause them to deform.

Is Luria broth defined or complex? Name one of the carbon and energy sources in the media.

For the three individual components state whether they are defined or undefined.

Tryptone:

Yeast extract:

NaCl:

ii) Pouring Luria agar plates

Materials:

- 40 mL Luria agar
- sterile petri dishes

Each student will be provided with 40 mL of molten Luria agar. This media has the same ingredients as the Luria broth above but 1.5% (weight/volume) agar has been added as a solidifying agent. Agar dissolves at 100˚C and so will dissolve during autoclaving (121˚C). The agar will not set until the temperature drops to below 40˚C. Agar can be kept in a 60˚C water bath to keep it molten before being poured into petri dishes.

Procedure:

1. Collect two petri dishes – take care when handling them as these dishes are sterile inside. Label the bottom of each dish with your name, tutor group and date. Place the dishes on the bench with the lid uppermost in front of a Bunsen burner.
2. Remove a 40 mL bottle of molten agar from the water bath. Take care when handling the molten agar as the bottles will be hot to handle. Use the silicon hand gloves provided.
3. Lift one side of the petri dish lid and carefully pour approximately 20 mL into each plate being careful not to produce any bubbles. Bubbles can be removed with a Bunsen flame. Ask your demonstrator how to do this if required.
4. Leave the plates for 30 minutes to set completely then give them to your demonstrator for storage. Your plates will be stored at 4˚C for use in next week's class.

What weight of agar (in grams) would be added to 40 mL Luria broth to give a 1.5% (w/v) concentration?

How do you think petri dishes are sterilised?

Why do we label the bottom of the petri dish?

3. MICR2011 course project

Whilst each weekly practical class involves independent laboratory activities, one exercise runs throughout the session and forms the basis of a total of 24% of the course assessment. In essence, the course project makes a comparison between traditional cultivation based microbiology and contemporary molecular microbiology. In your mini-group of four students will:

1) select an environment of interest
2) extract DNA from the environment of interest
3) identify microbes in that environment from DNA sequence data
4) isolate microbes from the environment of interest
5) identify microbes by DNA sequencing
6) identify your microbes using biochemical characterisation
7) identify your microbe using mass spectrometry
8) characterise antibiotic resistance property of your microbe

The project showcases the skills of a modern microbiologist from making media for cultivating microbes to bioinformatics based analysis of sequence data. You will be required to justify your choice of environment at the beginning of the session and produce a scientific research article describing your findings at the end of the session. The project justification is effectively an introduction to your research article. Typically an introduction consists of a claim of centrality (why it is important enough to spend time and money investigating), identification of knowledge gaps (what is not known about the microbiology of the environment you have chosen to investigate) and how you will fill the knowledge gaps. The final project manuscript should be modeled on published research articles in the *Journal of Bacteriology*. Understand the type of content appropriate for the methods, results and discussion sections. Communicating clearly and concisely is the key to good grades. A rubric for marking the final report will be provided on the Moodle page.

The main project for this course involves the selection of a particular environment of your choice for the study of its indigenous microbiota. By the end of the lab you must make your choice. Discuss possible environments with the other members in your mini-group and with your demonstrator. Consider how universal the interest in an environment is, and the experimental feasibility.

You will have materials to extract DNA from:

- Solid environments such as soil or food
- Aqueous environments such as seawater or pond water
- Surface environments such as skin or benches collected by swabbing

IMPORTANT! Once you have decided your sample, **add information about your sample to the OneNote class register** so that materials can be allocated for next week.

Complete by the end of this practical

Register your course project group using the MICR2011 Project Group Database Form Entry, you will need to provide:

1) Project Group Number (obtained when you list your group to the Table in Collaborative Space in OneNote Class Notebook)

2) Names of your project group members

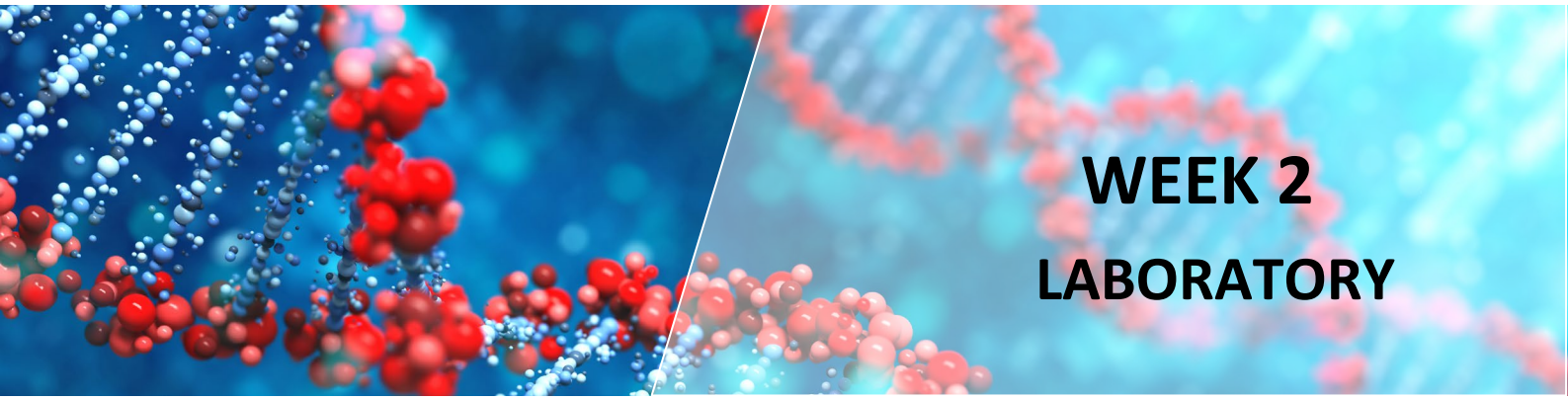
3) Pass for your newly created project group page in Collaborative Space (follow instruction provided in the Notebook).

4) Project sample type – solid OR aqueous OR surface

Complete for the Week 3 practical

Write a 500 word rationale for studying the environment of choice. Describe why the environment is significant and/or scientifically interesting. This will be due at the beginning of the Week 3 laboratory class through Moodle. Feedback on your Rationale will be returned in Week 4 and will help you write the introduction section of your draft and final report.

END OF WEEK 1



WEEK 2 LABORATORY

OVERVIEW

This week we begin the course project. We will take two approaches to studying the microbial communities (microbiomes) of the samples you have chosen. The first is a contemporary molecular approach and the second is a traditional cultivation approach. The molecular approach involves extracting DNA directly from the sample and using DNA sequencing technology to determine what microorganisms are present. We will perform the DNA extraction today and analyse sequence data in subsequent weeks. The cultivation approach involves growing individual microbial lineages present in the sample and subsequently characterising them using biochemical tests, microscopy, and DNA sequencing. We will inoculate growth media today and do some cell staining and microscopy training exercises.

LEARNING OBJECTIVES

- Understand the difference between modern DNA sequencing-based techniques and traditional cultivation-based techniques for identification microorganisms.
- Understand the advantages and disadvantages of each approach.
- Develop skills for visualising microbial samples by light microscopy.
- Be able to Gram stain a bacterial sample and understand the basis of the stain.

ACTIVITIES

1. DNA extraction of project samples for sequencing.
2. Staining bacteria for observation by light microscopy.
3. Inoculating liquid broth and agar plates with student samples.

Contents:

WEEK 2 RISK ASSESSMENT 35
WEEK 2 LABORATORY CLASS 36
1. DNA extraction of project samples for sequencing 36
Procedure 36
Materials 37
Step One - Sample collection and preparation 38
Solid samples (soil, food etc.) 38
Aqueous samples (seawater, pond water etc.) 38
Surface swab samples (skin, ear, benches etc.) 39
Step Two – Lysis 39
Step Three - Clean up (solid samples only) 39
Step Four - DNA binding and column washing 39
Step Five – Elution 40
Sequencing 40
2. Introduction to light microscopy 41
Parts of a light microscope 41
Setting up the light microscope 42
Resolution 44
Oil immersion 45
Microscopy trouble shooting 45
3. Staining bacteria for observation by light microscopy 46
Materials 47
Procedure 47
Smear preparation 47
Gram staining your heat fixed bacterial smear 49
Observations 49
4. Inoculating agar plates to obtain pure cultures and liquid broths as enrichment cultures 50
Materials 50
4.1 Procedure for inoculating samples to obtain single colonies (pure cultures) 50
4.2 Procedure for setting up enrichment cultures 50
Technique for subculturing agar plates for single colonies 51

WEEK 2 RISK ASSESSMENT

BABS Teaching Laboratory	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk3
Student Risk Assessment		

Hazards	Risks	Controls
General	See below	**<u>At all times</u>** follow demonstrators instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (human samples, environmental samples) Glass slides/coverslips Bunsen burner	infection cuts burns	PPE (lab coats, closed in shoes & gloves as required) and Safety glasses when handling human specimens. Adhere to aseptic techniques. Dispose of any broken/used sharp/glass items immediately in Sharps containers provided. Never leave a lit Bunsen Burner unattended or reach over an exposed flame. Keep long hair tied back.
Electrical Equipment (microscope)	Electric shock/ electrocution Exposure to biological agents (see above)	Avoid water/spillages when working with electrical items.
Chemical (DNA extraction)	Irritant for skin and eyes skin or eyes, toxic if ingested.	PPE (lab coats, closed in shoes, safety glasses & gloves. SDS.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 2 LABORATORY CLASS

1. DNA extraction of project samples for sequencing

DNA extraction underpins many modern techniques for the identification of microorganisms. Whether from a pure culture of a bacterial pathogen or a complex microbial consortium such as a stool sample, knowing what microbes you are dealing with starts with DNA extraction. DNA extraction from any given sample involves breaking open the cells, separating the DNA from the cell debris (membranes and proteins) and purifying the DNA by removing remaining contaminants that might interfere with downstream processing such as PCR and sequencing (e.g. RNA, cell metabolites, environmental matrix contaminants). The following procedure describes what you need to do to get a DNA extract from your sample. In your project mini-groups (groups of four), follow the protocol carefully and provide a labelled DNA sample to your tutor once completed.

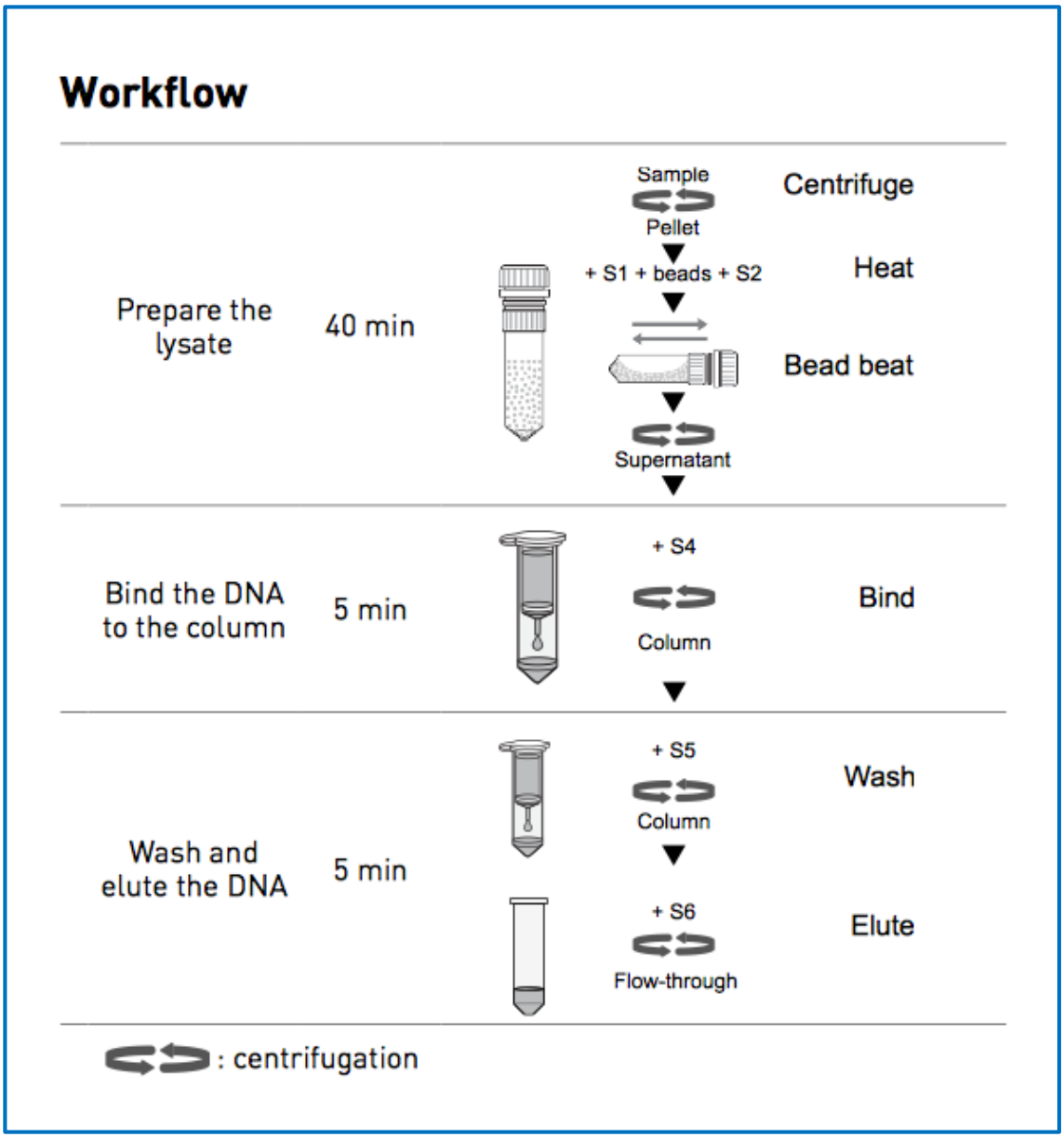
Procedure:

To isolate microbiome DNA from your sample, you will be using a commercial kit, the PureLink™ Microbiome DNA Purification Kit, which has been optimised for efficient microbiome DNA purification from a range of samples. Pay attention to the procedure needed for your particular sample as the protocols differ depending on the starting material. These procedures allow microbiome DNA isolation from the following three sample types:

- Solid environments such as soil or food
- Aqueous environments such as seawater or pond water
- Surface environments such as skin or benches collected by swabbing

There are four main steps to this DNA extraction protocol. The first is sample collection and preparation. Here the sample is collected from the environment and prepared for DNA extraction. Next is lysis, where the bacterial cells are broken open to release their DNA. This will be achieved by heating the samples in a lysis buffer and mechanically breaking open the cell walls by vigorously shaking the sample with glass beads. After this step, some samples will require an extra clean up step, which eliminates compounds that may inhibit the DNA sequencing process. DNA binding follows when the sample is added to a spin column that contains a filter that will bind the released DNA, allowing contaminants to be washed away by subsequent flushing of the column.

The DNA is then eluted by addition of an aqueous buffer that solubilises the DNA. The purified DNA is then ready for analysis by sequencing or PCR. An overview of the workflow is illustrated below. Discuss the procedure within your group. Assign tasks to each group member.



Materials:

- PureLink Microbiome DNA purification kit
- Forceps, blades, swabs, saline solution, mortar and pestle
- Pipettes, tips, microfuge tubes, microcentrifuges and vortexes

Step One - Sample collection and preparation:

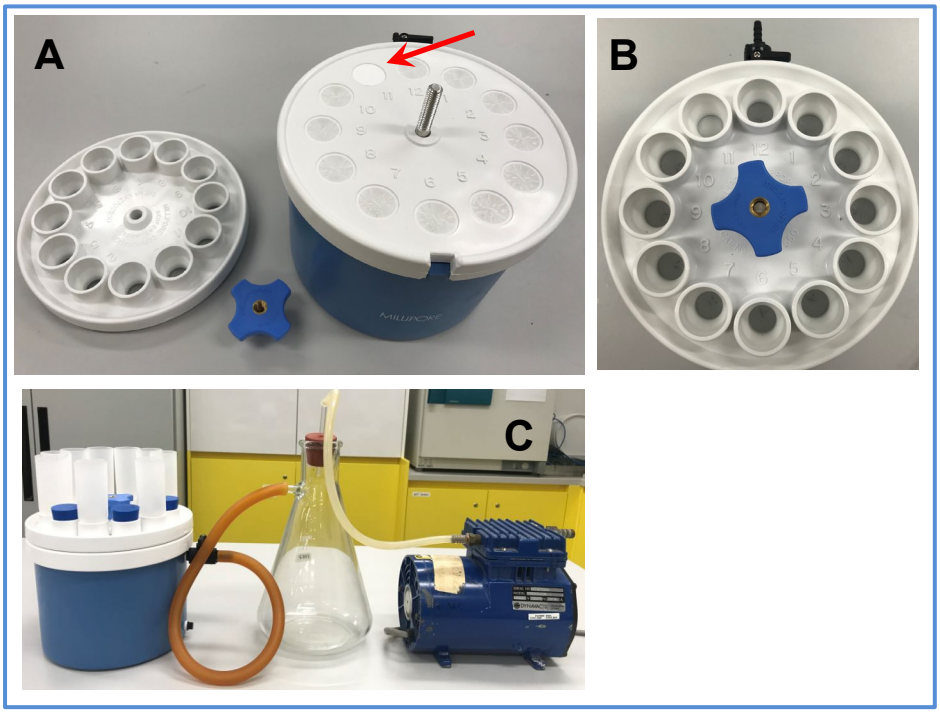
The method used to collect and prepare your sample will depend on the nature of the sample. Follow the appropriate guide for your sample type.

Solid samples (soil, food etc.)

i. Add 600 µL of Buffer S1 – lysis buffer to a bead tube (with red screw cap)
ii. Weigh out approximately 0.2 grams of your sample using the balance provided and add it to the bead tube. For food samples, it may be necessary to crush the sample using a mortar and pestle first.

Aqueous samples (seawater, pond water etc.)

i. Unscrew the blue nut on the top of the Vacuum Filtration Unit and remove the lid
ii. Remove a filter from its package using filter and place the filter in a container of deionized water to wet it completely.
iii. Remove the filter from the water using the forceps or soft-nosed tweezers and center it on a support screen. Make sure that the filter completely covers the screen.
iv. Using forceps, place a nitrocellulose filter disk onto the manifold (**A**) taking note of the position (1-12).
v. Replace the lid on the manifold and tighten the blue locking nut (**B**)
vi. Place stoppers in any positions around the manifold that do not contain filters. Add barrel extensions (max volume 50ml).
vii. Switch on the vacuum pump and filter the first 50ml of your sample, add more as the sample is filtered. Turn of the manifold and remove the lid (as in step i).
viii. Add 800 µL Buffer S1 – lysis buffer to a bead tube (with red screw cap) and add the filter membrane.



A: Setting the filter onto the manifold.
B: Filter loaded into the manifold.
C: Manifold with stoppers and barrel extensions connected to the vacuum pump.

Surface swab samples (skin, ear, benches etc.)

I. Add 600 µL of Buffer S1 – lysis buffer to a bead tube (with red screw cap)
II. Moisten the tip of a sterile cotton swab in sterile saline.
III. Swab the surface to be sampled thoroughly, making sure to use all sides of the swab.
IV. Place the tip of the swab into the bead tube and cut/break off the remainder. Ensure that any remaining handle does not prevent you from being able to cap the tube.

Step Two – Lysis

I. Label your bead tube with your group's identification on the side of the tube.
II. Add 100 µL of Buffer S2 – lysis enhancer to the bead tube. Cap tightly and mix tube contents briefly using the vortex mixer.
III. Incubate the tube in a heat block at 65°C for ten minutes.
IV. Homogenise the sample by shaking on a bead beater for 3 x 45 second cycles. *(Note: This step will be performed by the technical staff)*
V. Proceed to Step three (in the box below) if you are extracting from a solid sample. For all other samples proceed directly to Step Four.

Step Three - Clean up (solid samples only)

I. Centrifuge the tube containing your sample at 14,000 x *g* for 5 minutes.
II. Transfer up to 400 µL of supernatant to a clean microcentrifuge tube. Take care to avoid disturbing the pellet or transferring any debris.
III. Add 250 µL of Buffer S3 – Cleanup buffer to the supernatant and vortex immediately.
IV. Incubate on ice for ten minutes. Proceed to DNA binding (Step Four).

Step Four - DNA binding and column washing

I. Centrifuge the tube containing your sample at 14,000 x *g* for 2 minutes.
II. Transfer up to 500 µL of supernatant to a clean microcentrifuge tube, taking care to avoid disturbing the pellet or transferring any debris. Discard the tube containing the *pellet*.
III. Add 900 µL of Buffer S4 – Binding buffer to the supernatant and vortex briefly.
IV. Open the DNA binding column (supplied in a collection tube) and add 700 µL of your sample. Cap the column, place the column-tube assembly into a microcentrifuge and centrifuge at 14,000 x*g* for 1 minute.
V. Remove the column from its collection tube and discard the flow-through. Replace the column in its tube and repeat the previous step with the remaining amount of your sample.
VI. After the second spin, transfer the column (with DNA now bound to the filter inside) to the clean collection tube provided.
VII. Add 500 µL of Buffer S5 – Wash buffer to the column, cap it and centrifuge for 1 minute as above.
VIII. Discard the flow-through, replace the column in the tube and centrifuge for an additional 1 minute (this step removes residual wash buffer from the column).

Step Five – Elution

I. Transfer the column to a clean microcentrifuge tube labelled with your group identification.
II. Add 50 µL of Buffer S6 – Elution buffer to the column. Cap the column and allow it to sit at room temperature for one minute.
III. Centrifuge the column (in its microcentrifuge tube) at 14,000 x *g* for 1 minute. The DNA will now be eluted from the column into the tube, and is ready for quality analysis and sequencing. Hand your DNA extract to your demonstrator.

Sequencing:

The DNA extracted from your sample will be submitted to the Ramaciotti Centre for Genomics for further analysis. The quality of your DNA samples will be checked by gel electrophoresis and the quantity determined with a Nanodrop UV-Vis spectrophotometer. Those DNA extracts of sufficient quality and quantity will be used as template in a polymerase chain reaction that will amplify (make multiple copies of) a portion of the SSU rRNA genes present in your DNA samples. The product of the PCR (amplicons) will then be subject to sequencing using the Illumina MiSeq platform.

If you would like to learn more about Illumina sequencing technology (sequencing by synthesis) then go to:
www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf

What is/are the advantage(s) of using DNA sequencing-based methods for identification of microorganisms in your sample?

What is a disadvantage of only using DNA sequencing-based methods for identifying microorganisms?

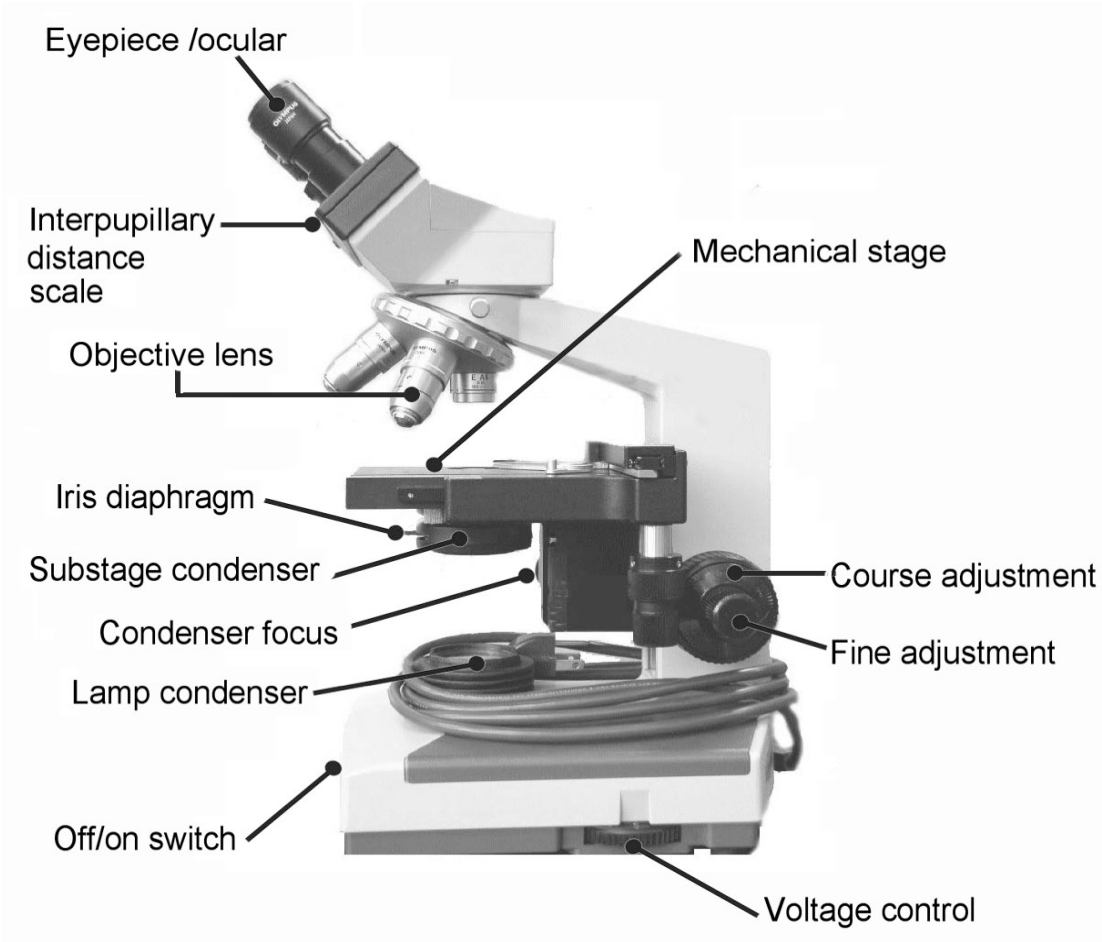
2. Introduction to light microscopy

The compound light microscope is an optical instrument designed for producing magnified images of objects using two or more glass lenses. The term light refers to the method by which light transmits the image to your eye. C*ompound* deals with the microscope having more than one lens. *Microscope* is the combination of two words; “micro” meaning small and “scope” meaning view.

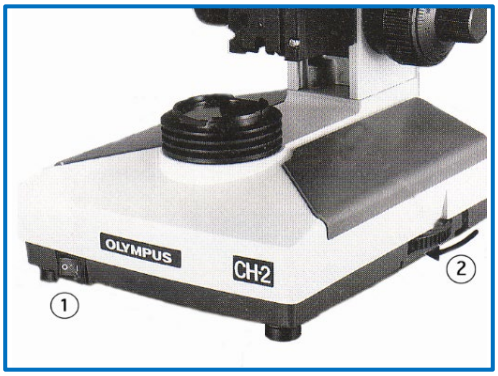
The size of organisms and the internal structures to be observed determine the type of microscopy needed. Use of the oil immersion objective (100x) is required if structures as small as 0.2 µm are to be observed. In this course students are required to demonstrate correct use of a light microscope with all lenses and appropriate illumination.

Parts of a light microscope

There are many makes and models of light microscope. However, all light microscopes are fundamentally the same, have similar controls and functions.
The microscope illustrated opposite is typical of the light microscope used in UNSW teaching



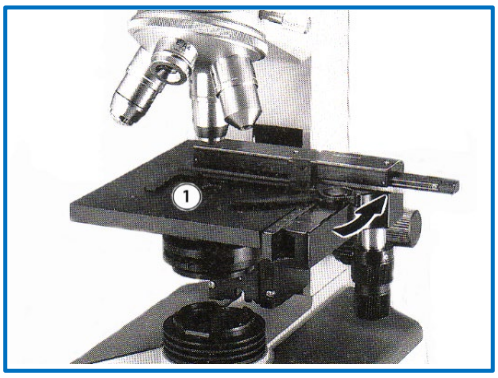
Setting up the light microscope:



1. Switching on microscope

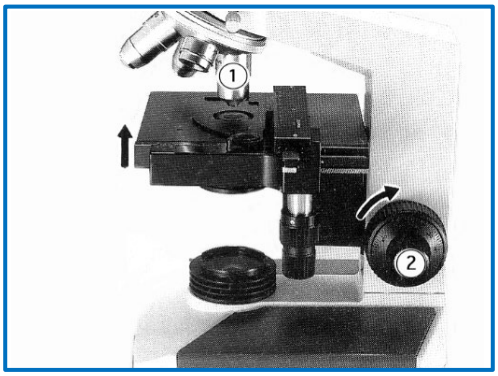
Turn the power switch on.

Rotate the voltage control clockwise to reduce light intensity or counter clockwise to increase intensity.



2. Specimen placement

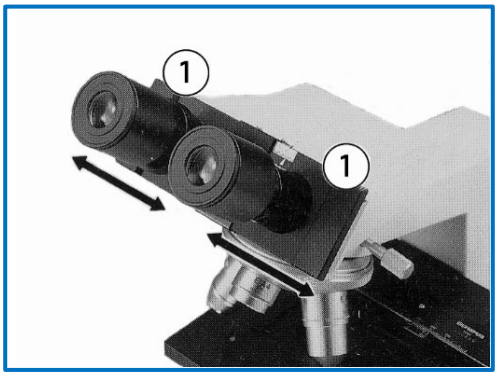
Open the spring-loaded finger of the specimen holder and insert the slide.



3. Focus

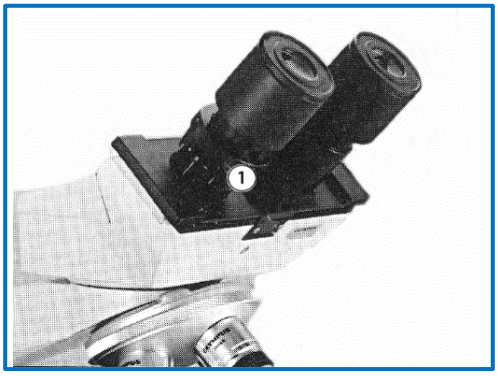
Swing in the 10x objective .

Bring the specimen into focus using the course and fine adjustment knobs .



4. Interpupillary distance

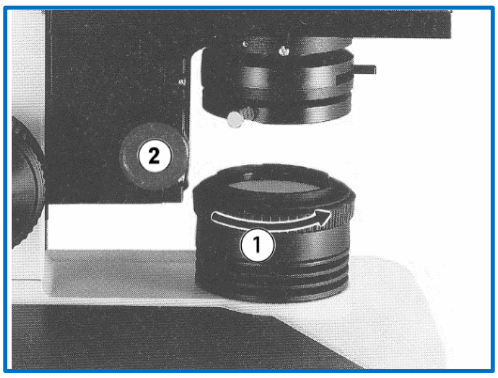
Looking through the binocular tube, move the knurled dovetail slides until a suitable binocular vision is obtained.



5. Dioptre adjustment

Look at the image through the right eyepiece with your right eye, and focus on the specimen with the fine focus adjustment.

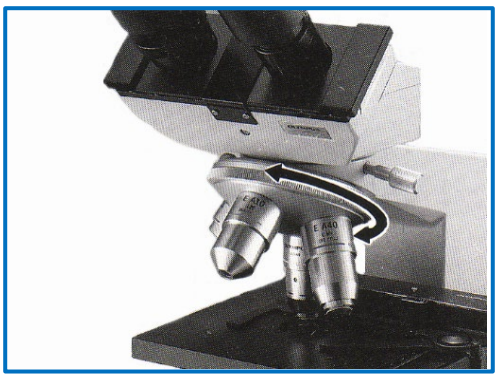
Looking at the image through the left eyepiece with your left eye, rotate the dioptre adjustment ring to focus on the specimen without using the focus adjustment knobs.



6. Centration of field iris diaphragm

Rotate the diaphragm ring counter clockwise to stop down the iris diaphragm to minimum.

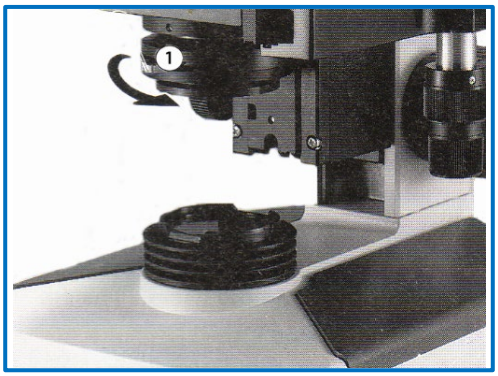
Rotate the condenser height adjustment knob in either direction until the image of the field diaphragm is visible sharply in the field of view.



7. Objective change

Swing in the desired objective for use.

Ensure that the nosepiece is clicked into position.



8. Aperture iris diaphragm adjustment

Turn the diaphragm lever counter clockwise to reduce diaphragm opening.

Resolution

A lens magnifies by bending light. Optical microscopes are restricted in their ability to resolve features by a phenomenon called diffraction which, based on the numerical aperture (NA or A_N) of the optical system and the wavelengths of light used (λ), sets a definite limit (d) to the optical resolution. Assuming that optical aberrations are negligible, the resolution (d) is given by:

$$d = \frac{\lambda}{A_N}$$

Usually, a wavelength of 550 nm is assumed, corresponding to green light. With air as medium, the highest practical A_N is 0.95, and with oil, up to 1.5.

Due to diffraction, even the best optical microscope is limited to a resolution of 0.2 micrometres.

Oil immersion

The oil immersion lens (100X), when used with a drop of oil, prevents refraction or deflection of angled light from its straight path that would occur if the light were to pass at an angle from glass into air. The degree to which the light is refracted or bent by a substance is formulated as its refractive index. As you might expect, the numerical aperture of a lens, the light-function constant you used to calculate the resolution, is determined in part by the refractive index of the glass.

To prevent the light from being bent away on an angled path from the objective lens, allowing the maximum amount of light from the specimen to be gathered by the objective, a drop of immersion oil may be placed on the specimen and the oil immersion objective then lowered into the oil.

Immersion oil has the same refractive index as the glass so light travelling up through the slide, the oil and the objective lens is not refracted again until it passes from the convex upper surface of the lens into the air above. That bending, however, is what the lens is designed to do, sending the rays which left the specimen at angles up the tube at new coherent angles to be resolved and magnified by the ocular lens.

As light strikes the specimen the qualities of the light are changed in several ways that give the visual image we perceive. It may be scattered or reflected away from a path leading to the objective, darkening the image; it may be completely occluded by solid structures that appear black to the observer; specific wavelengths of the light may be partially absorbed by certain substances (including stains), giving a characteristic colour to structures containing them.

Microscopy trouble shooting

Occasionally you may have trouble with working your microscope. The following are some common problems and solutions.

- Image is too dark!
  Adjust the diaphragm, make sure your light source is on.

- There's a spot in the viewing field, even when I move the slide the spot stays in the same place!

Your lens is dirty. Use lens paper, and only lens paper to carefully clean the objective and ocular lens. The ocular lens can be removed to clean the inside.

- Can't see anything under high power!
  Remember the steps, if you can't focus low power, you won't be able to focus anything under high power.

- Only half of the viewing field is lit, it looks like there's a half-moon in there!
  You probably don't have your objective fully clicked into place

Apparent fault	Possible cause	Correction
Field dark	Power (lamp) not on or turned down too low	Turn power on & check voltage
	Condenser diaphragm closed	Open diaphragm
	Lamp filament burnt out	Replace lamp
Colour of objects indistinct	Condenser diaphragm closed too far	Open diaphragm
Poor resolution	Condenser either too far open or too far closed	Adjust condenser diaphragm
Unable to focus on object	Cover-slip too thick	Replace
	Slide up-side down	Invert slide
	Focusing attempts too rapid	Use fine focus and adjust more slowly
	Objective has insufficient resolving power	Use higher power
	Objective covered with dried immersion oil from previous use	Clean with lens tissue and solvent
Specks in field of view	Dirt on eye lens of ocular	Clean with lens tissue
	Dirt on condenser lens	
	Dirt on filter	
Moving shadows in field	Air and/or water bubbles in immersion oil	Remove oil with lens tissue. Re-apply
Light suddenly reduced	No oil contact between oil immersion objective and slide	Adjust with course /fine focus control

3. Staining bacteria for observation by light microscopy

Unstained cells are nearly transparent when observed by light microscopy and hence are difficult to see. However, most microbes can be readily stained.

The Gram stain is the most important differential stain in bacteriology. The majority of bacteria can be classified as Gram positive or Gram negative, a characteristic that is produced by marked differences in cell wall structure and correlates with many other characteristics of the microorganism.

Materials:

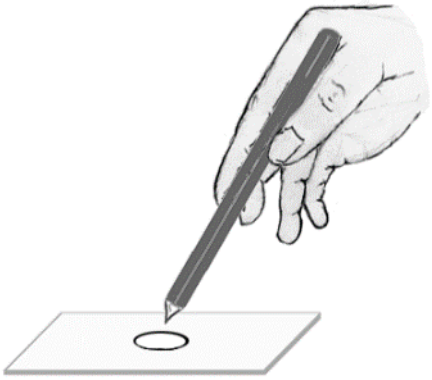
- Three broth cultures, one pure culture of a Gram-positive organism, and one pure culture of a Gram-negative organism, and one unknown.
- Gram reagents.

Procedure:

The Gram stain is the most important differential stain in bacteriology. All bacteria can be classified as Gram positive or Gram negative, a characteristic that is produced by marked differences in cell wall structure and correlates with many other characteristics of the microorganism.

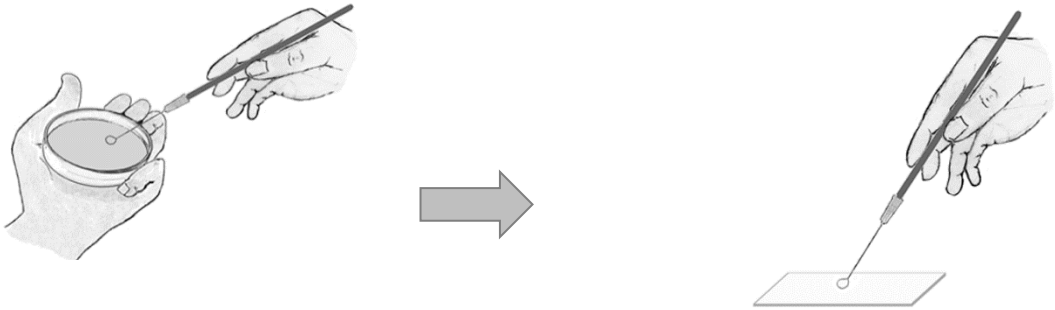
Smear preparation:

i. Obtain a clean glass microscope slide. Using an indelible marker such as a CHINAGRAPH® pencil, draw a small circle on the slide where you intend to locate the smear.



ii. Sterilise loop by flaming in Bunsen burner as previously described (bacteriological loop method).

iii. Using aseptic technique, collect a portion of a single colony with the loop and mix with the water on the slide to provide a shallow, uniform and slightly turbid layer. If staining organisms from solid media, apply a loop-full of sterile distilled water to the circled area of the slide. (Organisms from liquid or broth culture may be applied to the slide directly).



iv. Allow the smear to air dry. When smear has completely dried, heat fix by passing underside of slide rapidly through the Bunsen flame two or three times. Allow to completely cool before applying any stains.

Gram staining your heat fixed bacterial smear:

i. Flood air-dried, heat-fixed smear of cells for 1 minute with crystal violet staining reagent. Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

ii. Wash slide in a gentle and indirect stream of water for 2 seconds.

iii. Flood slide with the mordant: Gram's iodine. Wait 1 minute.

iv. Wash slide in a gentle and indirect stream of water for 2 seconds.

v. Flood slide with decolorising agent (alcohol). Wait 15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.

vi. Flood slide with counterstain, safranin. Wait 30 seconds to 1 minute.

vii. Wash slide in a gentle and indirect stream of water until no colour appears in the effluent and then blot dry with absorbent paper.

viii. Observe the results of the staining procedure under oil immersion using a light microscope. At the completion of the Gram Stain, Gram-negative bacteria will stain pink/red and Gram-positive bacteria will stain blue/purple.

Observations:

Organism	Gram reaction	Morphology (cell shape)
Gram +ve	positive	
Gram -ve	negative	
Unknown		

What component of the bacterial cell determines the outcome of the Gram stain?

Why are cells counter stained at the end of the staining procedure?

4. Inoculating agar plates to obtain pure cultures and liquid broths as enrichment cultures

Most techniques for the isolation of bacteria involve streaking an aqueous suspension of cells onto agar medium (to dilute the inoculum and obtain single colonies) and incubating for colony development. If a cell can grow on the media it will replicate until ultimately there is a colony of cells visible to the naked eye. The steps taken to obtain pure cultures are usually carried out a number of times in succession to safeguard against the transfer of contaminants. Sub-culturing is a useful microbiological technique and competence in it is desirable. Check your method with your demonstrator. If your plates are not adequate, repeat the initial isolation step.

In some instances, the organisms you are trying to detect may be present in your sample in very low numbers and may not grow successfully on plates directly from the original sample. In such instances the original sample can be incubated in a broth culture that will promote the growth of organisms – i.e. an enrichment culture. This is particularly relevant when trying to detect small numbers of possibly pathogenic organisms in human samples, especially blood cultures, or food samples or isolating microbes from environmental samples with specific abilities (e.g oil degrading bacteria from soil samples).

Materials:

- Samples of your choice for course project
- Luria broth - that you prepared in last week's class
- Agar plates:
    - Luria agar – plates you prepared in last week's class
    - LB20 – Luria agar with additional sodium chloride for marine samples
    - M9 – minimal salts agar with glucose as a carbon and energy source

4.1 Procedure for inoculating samples to obtain single colonies (pure cultures):

Each group will make a suspension of their sample and every student will then culture this onto the Luria plates you prepared last week and the M9 plates supplied. For those students working with marine samples, also plate out on LB20, which has an elevated NaCl concentration closer to that of seawater.

Label the bottom of your agar plate with your name, date and group number. Also write a suggested incubation temperature on the plate.

Using the 16 streak technique described on page 36, inoculate your sample suspensions onto the agar plates.

Give the plates to your demonstrator for incubation. Check the plates next week for colony formation. The colonies will be your project microbes that you will nurture and characterize throughout the rest of the course and ultimately identify.

4.2 Procedure for setting up enrichment cultures:

Aseptically transfer 200 µL of your sample suspension into the Luria broths you prepared last week.

Label the broths with your name, date and demonstrator's name and give them to your demonstrator. Suggest an incubation temperature (room temperature, 30ºC, or 37ºC).

Don't despair if you obtain no colony growth on your primary isolation agar plate (3a). Next week you can subculture your enrichment broth using the procedure above.

Technique for subculturing agar plates for single colonies:

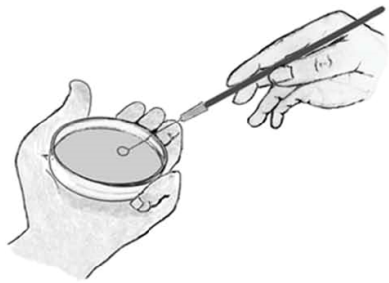
A

The bacteriological loop is sterilised by flaming in a Bunsen burner and allowed to cool. Care is required at all times so that the loop remains sterile (**A**).

Collect a portion of microbial growth with the sterile loop.

Working in close proximity to the flame, pick up the base of the new culture plate with your free hand and hold it midway between a vertical and horizontal position (**B**).

B

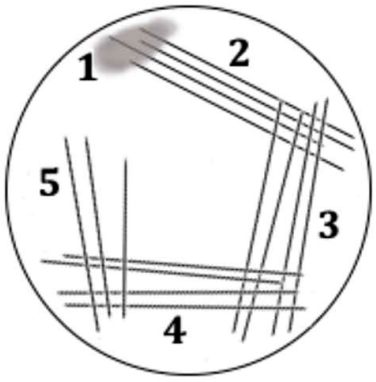


Transfer the culture by carefully smearing the inoculum onto the top section of the plate (**C1**). Take care not to cut into the agar.

Replace the plate onto its lid. Flame the loop and allow to cool.

Pick up the base of the culture plate again and locate the area previously inoculated. Using the sterile loop, make 3 - 4 streaks out from the original inoculum onto the adjacent section of the agar plate (**C2**).

C



Replace the plate onto its lid. Flame the loop and repeat the above process 2 – 3 times more so that the agar plate is inoculated around its perimeter (**C3 & C4**).

The final streak can be directed toward the centre of the plate. Take care not to touch the original inoculum on the plate (**C5**).

Suggestive learning activity:

Weekly group discussion for your course project - check Collaboration Checklist for suggestive topics

Don't forget to record group meeting minutes (meeting minute template) and develop weekly action plan (And stick to the plan!).

END OF WEEK 2

WEEK 3
LABORATORY

OVERVIEW

Isolation of microorganisms is required to unambiguously assign a property to the organism. Fulfilling Koch's postulates requires isolation. Phenotyping is a traditional means of identifying microbes in medical and food diagnostics. This week we will be checking out what grew on our plates from our samples, considering the purpose of enriched, selective and differential media, performing some phenotypic characterisation and learning how to count microorganisms.

LEARNING OBJECTIVES

- Understand what enriched, selective, and differential media are and how they can be used to isolate specific microorganisms.
- Understand the basis for some common biochemical tests that can be used to characterise a microorganism.
- Be able to perform a viability count and calculate cell density from the results.

ACTIVITIES

1. Examination of cultures obtained from your samples from Week 3.
2. Enriched, selective and differential media.
3. Biochemical tests used for phenotypic characterization.
    a. Catalase.
    b. Oxidase.
    c. Indole.
4. Viable counts of microorganisms.
5. Submit your Rationale for your Sample of Choice to your demonstrator

Contents:

WEEK 3 RISK ASSESSMENT 55
WEEK 3 LABORATORY CLASS 56
1. Examination of cultures obtained from project samples 56
2. Selective and differential media 56
Materials 56
Procedure 57
Examples of some commonly used growth media 57
Nutrient agar (NA) 57
Blood agar (sheep or horse - SBA or HBA) 57
MacConkey agar (MAC) 58
Sabouraud agar (SAB) 58
Mannitol salt agar (MSA) 58
Questions 58
3. Biochemical tests used for phenotypic characterisation 62
3.1 Catalase 63
Medium 63
Test 63
Interpretation 63
Materials 64
Procedure 64
Results 64
3.2 Oxidase 64
Test 65
Materials 65
Results 65
Questions 66
3.3 Indole 67
Materials 67
Procedure 67
Results: 68
Questions 68
4. Viable counts of microorganisms 69
Materials 70
Procedure 70
Dilution steps 70

WEEK 3 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk4

Hazards	Risks	Controls
General	See below	**At all times** follow demonstrators' instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (Microorganisms, Risk Group I and Risk Group II) Glass slides/coverslips Bunsen burner	infection cuts burns	PPE (lab coats, closed in shoes & gloves as required). Adhere to aseptic techniques. Work in PC2 laboratory or BSC if required. Dispose of any broken/used sharp/glass items immediately in Sharps containers provided. Never leave a lit Bunsen Burner unattended or reach over an exposed flame. Keep long hair tied back.
Chemical (hydrogen peroxidase & indole reagent, gram stain)	corrosive, irritant to eyes, respiratory system and skin	PPE (lab coats, closed in shoes, safety glasses & gloves. SDS.
Electrical Equipment (microscope, vortex)	Electric shock/ electrocution Exposure to biological agents (see above)	Avoid water/spillages when working with electrical items.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 3 LABORATORY CLASS

1. Examination of cultures obtained from project samples

Examine your plates from last week and select isolated colonies for further examination and subculture. Sub-culturing isolates will be a routine exercise until Week 8. If adequate biomass has formed on your plates or in your broth, Gram stain the colonies you have sub-cultured using the protocol described in last weeks' class.

Save and store photos of your plates. Record your Gram stain results here (you'll need these for your final report):

Suggestive learning activity:

Weekly group discussion for your course project - check Collaboration Checklist for suggestive topics

Don't forget to record group meeting minutes (meeting minute template) and develop weekly action plan (And stick to the plan!).

2. Selective and differential media

Specialised media can be used to enrich, isolate and differentiate between different microbes. Enriched media often contain complex substrates such as blood, serum or brain heart infusion that promote the growth of fastidious (fussy) organisms. Selective media are formulated to grow only certain microbes while inhibiting the growth of others. Differential media allow for the differentiation of bacteria based on their biological characteristics such as the fermentation of a particular sugar. Some specialised media can be a combination of two of the above characteristics. For example, MacConkey and Mannitol Salt Agars are both selective and differential and Horse blood agar is enriched and differential.

Materials:

- Photograph of a MacConkey agar plate with (per tutor group):
  - *Escherichia coli*
  - *Proteus mirabilis*
- Photograph of a Mannitol salt agar plate with (per tutor group):

- *Staphylococcus epidermidis*
- *Staphylococcus aureus*

- Photograph of a Horse blood agar plate with (per tutor group):
  - *Streptococcus pyogenes*
  - *Streptococcus pneumoniae*

Procedure:

Examine all photographs of plates and complete table showing amount of growth and reaction for each organism.

Organism	Specialised agar	Growth and Appearance
S. epidermidis		
S. aureus		
E. coli		
P. mirabills		
S. pyogenes		
S. pneumoniae		

Examples of some commonly used growth media:

Nutrient agar (NA)

A basal medium containing peptones, yeast extract and sodium chloride.

Blood agar (sheep or horse - SBA or HBA)

This enriched medium contains whole red blood cells and supports growth of most pathogenic bacteria and yeast. It is also a differential medium.

- ß-haemolysis (complete haemolysis – i.e. a clear zone around the bacterial colonies): *Streptococcus pyogenes.*
- α-haemolysis (partial haemolysis - i.e. a greenish zone around the bacterial colonies): Viridans streptococci, e.g. *Streptococcus sanguis, Streptococcus bovis, Streptococcus mutans*, *Streptococcus pneumonia.*

Most other organisms grow well but any haemolytic effect shown is irrelevant for differentiating between bacterial isolates, e.g. some strains of *Staphylococcus aureus* produce lysis while other strains show no lysis at all.

MacConkey agar (MAC)

Contains bile salts that inhibit many non - enteric organisms making it a selective medium. It also contains the carbohydrate lactose and the pH indicator neutral red, allowing differentiation between lactose fermenting and non-fermenting lineages. This makes it an indicator or differential medium.

- Lactose fermentation positive (pink/red colonies): *Escherichia coli, Klebsiella* sp.
- Non-lactose fermentation (creamy colonies): *Proteus* sp., *Shigella* sp., *Salmonella* sp., *Pseudomonas aeruginosa*.

Sabouraud agar (SAB)

Contains a high concentration of glucose at a low pH that inhibits bacteria. Used for the isolation of yeast and fungi.

Mannitol salt agar (MSA)

Contains a high concentration of sodium chloride (7.5%) that is selective for *Staphylococcus* sp. Also included in the medium is the pH indicator phenol red and the carbohydrate mannitol. *Staphylococcus aureus* grows luxuriantly, ferment the mannitol, and produces colonies with yellow zones. Non-pathogenic Staphylococcus lineages produce small colonies with no colour change of the surrounding medium. Other bacteria are generally inhibited.

Questions:

What is the media component that makes MacConkey Agar selective? Explain.

What is the media component that makes MacConkey Agar differential? Explain.

What is the media component that makes Mannitol Salt Agar selective? Explain.

What is the media component that makes Mannitol Salt Agar differential? Explain.

Predict what growth would be seen on a MSA plate inoculated with *Staphylococcus epidermidis* and incubated anaerobically?
Explain.

What is the media component that makes Horse Blood Agar an enriched agar? Explain.

What is the media component that makes Horse Blood Agar differential? Explain.

Biochemical tests used for phenotypic characterisation

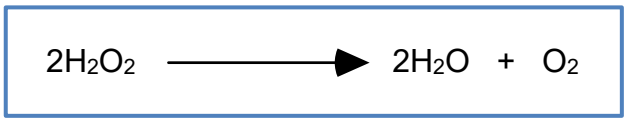
Staining bacteria and observation by light microscopy provides information about bacterial morphology, gram reaction, and potentially the presence of structures such as endospores. However, microscopic examination alone gives little information as to the genus and species of a particular bacterium.

To phenotype bacteria, some form biochemical analysis must be performed. The types of biochemical reactions each organism undergoes acts as a 'fingerprint' for its identification. Different species of bacteria have unique DNA and since DNA codes for protein synthesis, then different species of bacteria must, by way of their unique DNA, be able to synthesise different protein enzymes. Enzymes catalyse all the various chemical reactions of which the organism is capable. Therefore, different species of bacteria must carry out unique sets of biochemical reactions. By determining which enzymes are present in an unknown organism a description of that organism may be made and hence a step toward identification by phenotyping.

For example, all bacteria contain enzymes that can reduce oxygen to hydrogen peroxide and, in smaller amounts, to the more toxic free radical, superoxide. In most aerobes and facultative anaerobes, accumulation of these two toxic materials is prevented by the action of two enzymes, catalase and superoxide dismutase. Catalase is a hemoprotein enzyme of the oxidoreductase class that catalyses the conversion of hydrogen peroxide to water and oxygen. Superoxide dismutase is a copper-and zinc-containing enzyme present in all oxygen-using organisms that scavenges free radicals and converts them into H_2O_2 and O_2). Organisms that can tolerate exposure to oxygen always contain superoxide dismutase and may contain catalase. Lactic acid bacteria, being unable to synthesize heme molecules, do not contain catalase. Therefore, they are catalase negative. This group contains the enzyme peroxidase which catalyses the oxidation of organic compounds, such as $NADH_2$ by H_2O_2 which is thereby reduced to water. Lactic acid bacteria are therefore protected from H_2O_2 toxicity in the absence of catalase.
Enzymes are detected by the chemical reaction they carry out. However, it is necessary to have a means of observing and possibly measuring an enzyme's chemical reaction. One common measuring technique involves the reaction of a pH indicators that respond to the hydrogen ion, (H+), concentration by changing colour as a direct result of an enzyme's activity. The quantity of H+ in a solution is a measure of how acid or basic (alkaline) a solution is. Thus, if an enzymatic reaction produces acids, which increases the H+ concentration, pH-indicators respond by changing colour as the acidity changes.

3.1 Catalase

Catalase is an enzyme, containing haemin as the prosthetic group. It is widely distributed in nature, being present in most aerobic cells where its function is to remove the highly toxic peroxide (H_2O_2) that is formed in small amounts during passage of electrons down the electron transport chain to oxygen. Its presence may be tested by looking for the appearance of gas bubbles after the organism is added to hydrogen peroxide.



It is used to distinguish between morphologically similar families of bacteria e.g. distinction of the Gram-positive cocci which are members of the Micrococcaceae (catalase positive) from those belonging to the Streptococcaceae (catalase negative), or Gram-positive rods of the Coryneform group (positive) from those of the Lactobacillaceae (negative).

Medium

Any medium supporting growth can be used to obtain cultures for the catalase test with the exception of those containing blood. Blood agar may contain residual catalase sufficient to give a false positive result, and in addition, some streptococci, normally catalase negative, may be able to form catalase if the haem group is supplied by the blood.

Test

The catalase test can be performed in several different ways as outlined below:

(a) Place one drop of H_2O_2 (10 vols.) on a glass slide and emulsify in it a colony from an agar plate or slope.

OR

(b) Pour approximately 2 ml of H_2O_2 over the surface of a 24 hour agar slope culture and set the tube in an inclined position.

OR

Place one drop of H_2O_2 on a colony on agar medium and one drop, as a control, on the medium itself, which may contain sufficient catalase to give a false positive reaction (e.g. blood agar).

Interpretation

- Positive result ⇒ immediate production of bubbles of oxygen
- Negative result ⇒ no bubbles formed

Materials:

- Culture plate of *Staphylococcus*
- Culture plate of *Lactobacillus*
- Hydrogen peroxide

Procedure:
For each bacterial culture, place one drop of H_2O_2 (10%) on a glass slide.

Aseptically pick up a colony and emulsify in the drop of H_2O_2

Results:

Catalase reaction

Staphylococcus	
Lactobacillus	

What is the gaseous breakdown product of hydrogen peroxide?

3.2 Oxidase

Cytochromes are haem-containing proteins of high molecular weight and are involved in respiratory metabolism. The iron in the haem-moiety of cytochromes is responsible for the reduced or oxidized state of the cytochrome.
In the positive oxidase of cytochrome c-test, the cytochrome c must be oxidized

Electrons from the reduced cytochrome C are taken up by oxidized cytochrome oxidase which passes them to free oxygen molecules. In the oxidase test, tetramethyl-p-phenylene diamine is oxidized by oxidized cytochrome c to form a purple substance (Wurster's blue).

A number of bacteria give a positive oxidase reaction and it can be used to distinguish organisms such as members of Enterobacteriaceae (negative) from the Vibrionaceae (positive).

Medium

Grow organisms for the test on any appropriate medium.

Test

- Method 1: Pour a freshly prepared 1% aqueous solution of p-phenylene-diaminehydrochloride over colonies and immediately pour off.

Positive	⇒	Faint purple colony immediately.
	⇒	Reddish purple colony in 3-5 min
	⇒	Intense jet black colony in 30 min Note: Media may change colour after 30 min. This is not a positive reaction.
Negative	⇒	No colour change in colony

OR

- Method 2: Soak a filter paper with the reagent. Remove a portion of bacterial growth and rub onto filter paper using platinum loop. Nichrome loops must not be used because false positive reactions may be obtained because of oxidation of tetramethyl-p-phenylenediamine by nichrome.

Positive	⇒	Development of purple within 20 seconds
Negative	⇒	No colour.

Materials:

- Culture plate of *Enterobacteriaceae*
- Culture plate of *Vibrionaceae*
- Oxidase reagent (tetramethyl p-phenylene diaminehydrochloride)
- Platinum bacterial loop
- Filter paper

Results:

Oxidase reaction

Enterobacteriaceae	
Vibrionaceae	

Questions:

Can you explain why it is necessary to use a platinum loop?

Why it is necessary to set a strict time limit for observing colour change when carrying out an oxidase test?

Would you expect a strict anaerobe to be oxidase positive or negative? Explain.

3.3 Indole

Amino acids may arise as a result of the hydrolysis of proteins, peptones and peptides. Tryptophan is an amino acid that can be used as an energy generating substrate by certain genera of bacteria with in the Enterobacteriaceae. Such bacteria can hydrolyse tryptophan, by means of an enzyme tryptophanase (one of the products of this reaction being indole), which can be detected colorimetrically by a simple test. The reaction for detection of indole depends on the reaction between a pyrrole (e.g. indole = benzopyrrole) and a weakly alcoholic solution of p-dimethylaminobenzaldehyde producing a red-pink colour.

The gene encoding for tryptophanase (*tna* A in *E. coli*) is expressed only when tryptophan is present in the growth medium i.e. tryptophan is required for the induction of enzyme synthesis. In addition, it has been shown that glucose represses expression of the tryptophanase gene. It is essential therefore, when testing for the presence of tryptophanase to ensure that the growth medium contains tryptophan but not glucose. Usually a peptone broth containing 1% L-tryptophan is used.

ρ-Dimethylaminocinnamaldehyde (DMACA) is a sensitive indole reagent, capable of detecting 3 µg of indole per millilitre of medium. In fact, the reagent can be mixed with a small amount of bacterial culture to detect the presence of indole in the culture. Indole combines with DMACA to form a blue-green compound.

Materials:

- Culture plate of *Escherichia coli*
- Culture plate of *Proteus mirabilis*
- BactiDrop Spot Indole
- Filter paper

Procedure:

1. Dispense a drop of BactiDrop Spot Indole test solution onto a piece of filter paper.
2. Smear the growth from an actively growing pure culture onto the saturated filter paper.

3. Observe for the development of a blue colour within 3 minutes.

Results:

Indole reaction

E. coli	
Proteus mirabilis	

Questions:

What is the mechanism of indole test?

Why must glucose be excluded from the growth medium when performing the indole test?

4. Viable counts of microorganisms

The abundance of microorganisms plays a major role in how microbes impact humans, therefore quantifying or enumerating cells is important in microbiology. There are many ways to do this including by directly counting the cells using a microscope and indirectly by viable cell counts on agar plates or by alternative methods such as quantitative PCR, measurement of liquid culture turbidity or quantifying cell substances (e.g. protein, dry weight, total nitrogen). Be mindful of the fact that most of these methods will include dead cells in the final count.

A count of viable cells (capable of replication) is valuable when some kinds of microorganisms can be most conveniently distinguished by using a selective characteristic (e.g. presumptive coliform count in drinking water analysis). It is a useful skill for a microbiologist to be able to perform an accurate count of organisms present in a suspension.

Today you will learn how to do a viable count. There are three types of prerequisite skills required:

- The ability to estimate the concentrations of organisms in a culture to be counted.
- The ability to calculate the appropriate dilutions to culture.
- The ability to prepare the dilutions aseptically and to inoculate media appropriately.

This exercise allows each student to practice these three skills.

An overnight broth culture of the yeast *Saccharomyces cerevisiae* (prepared by technical staff) will be counted using the spread plate method. Aliquots (100 µL) of appropriate dilutions will be spread evenly onto the surface of nutrient agar plates.

NOTE: Calculations have to be made to ensure that between 30-300 organisms are likely to be in one of the dilutions. You don't want more than 300 colonies on your agar plate because it gets too crowded to count. You don't want less because small numbers don't accurately reflect reality.

Materials:

- An overnight culture of *Saccharomyces cerevisiae* in Sabouraud broth (test sample)
- A series of suspensions of *S. cerevisiae* in Sabouraud broth (reference samples with known cell concentrations)
- 15 mL of sterile saline (per pair of students)
- Microfuge tubes
- Sterile 1 mL pipettes tips
- Sterile plate spreaders
- 3 Sabouraud (SAB) agar plates (per pair of student)

Procedure:

Work in pairs.

Compare the test culture with the reference suspensions of *S. cerevisiae*. Estimate the approximate cell concentration of the test culture.

Estimate (cells/mL) =

Based on your estimate of cell concentration, calculate what dilution will result in 30-300 cells in 100 µL. For an accurate determination of viable cell counts you need 30 – 300 colonies per agar plate.

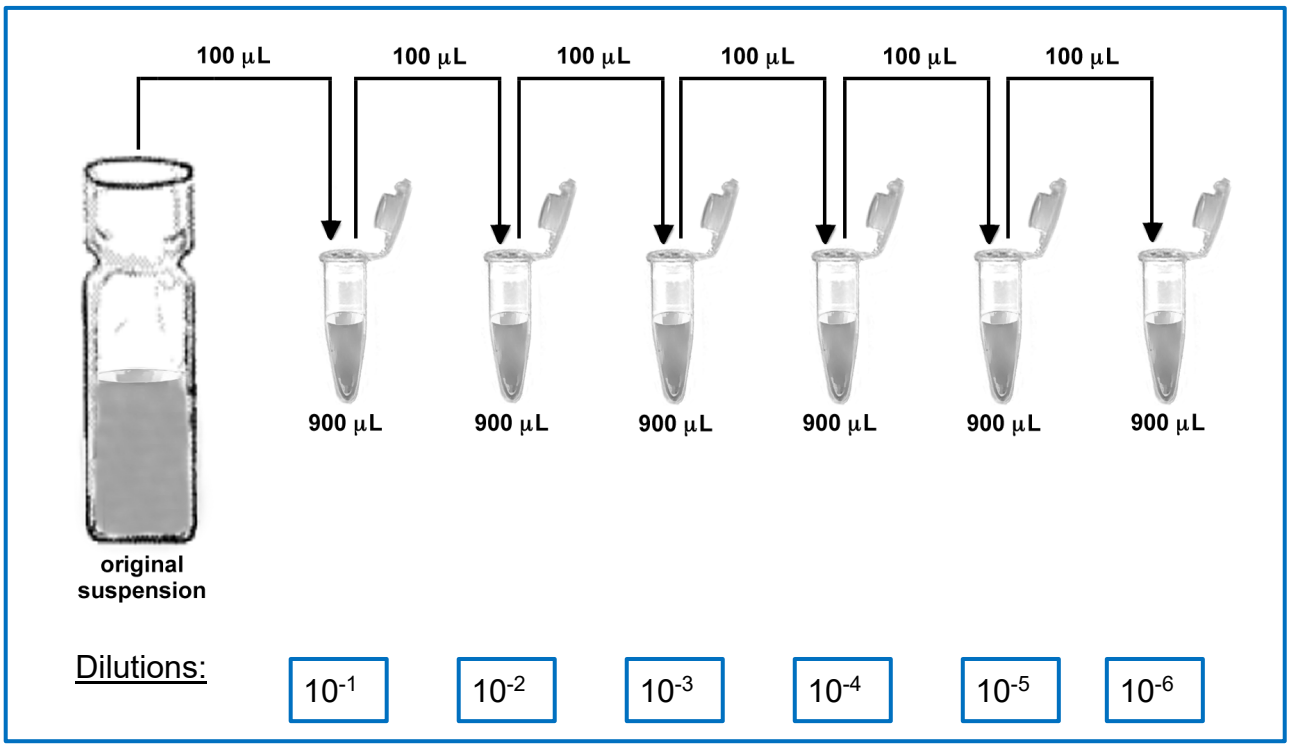
Decide what dilution steps are required to produce this cell concentration.

Prepare one set of dilutions of the test culture to suit your calculations using 900 µL of diluent (saline solution). (Note: a 1 in 10 dilution is 100 µL added to 900 µL).

With a new pipette tip for each step, transfer 100 µL into the first tube (10^{-1}).

Mix thoroughly and then transfer 100 µL from the first to the second tube (10^{-2}) and so on according to your calculations. See diagram below.

Dilution steps:



Show your calculations for the spread plates in the table below and check these with your demonstrator.

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Expected cells/mL								
Expected cells/100 µL								

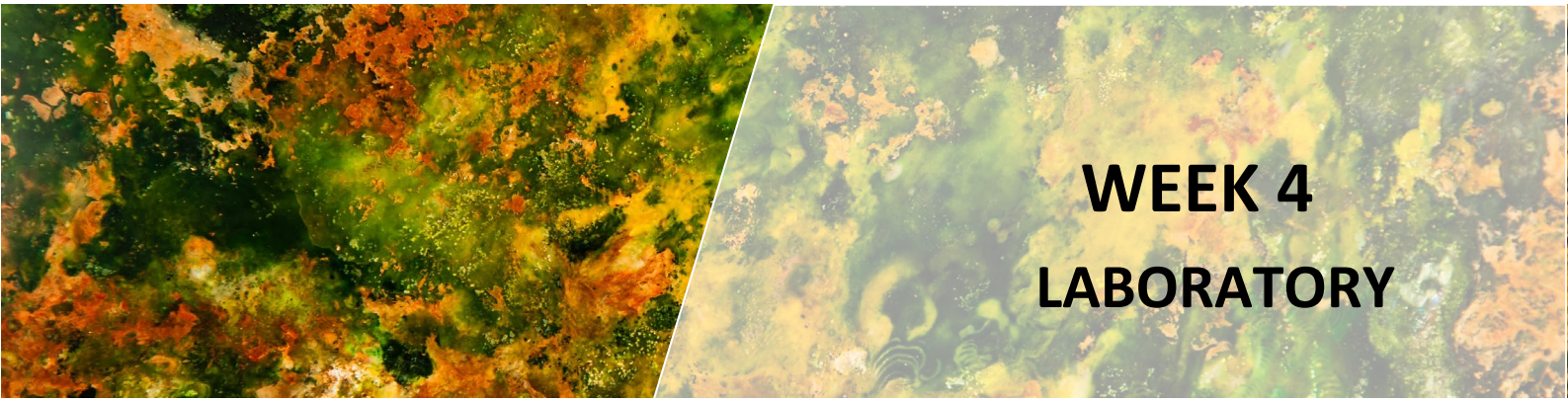
Label your agar plates with your name, date and dilution factor.

Aliquot a 100 µL sample of the dilution you have estimated will contain 30-300 cells onto one of your agar plates and spread the aliquot evenly over the surface of the plate using a sterile spreader.

Repeat the same with the dilutions one factor above and below the dilution used in the previous step on separate agar plates and spread as before. For example, if you estimated that your 10^{-3} dilution contained 30 - 300 cells then you should spread out dilutions 10^{-2}, 10^{-3} and 10^{-4} onto your 3 agar plates.

Give your labeled plates to your demonstrator for incubation. The viable cell count expressed as colony forming units per mL (CFU/mL) will be determined in next week's class.

END OF WEEK 3



WEEK 4
LABORATORY

OVERVIEW

In the tutorial and lectures this week you studied microbially driven nutrient and energy cycling. The simplest (and most classic) demonstration of how nutrients and energy are recycled in the environment is the Winogradsky column – discussed in the tutorial. The Winogradsky column demonstrates the development of a stratified microbial community that is inter-dependant, and where the waste of one microbe is the fuel for another. Unfortunately, a Winogradsky column takes about 8 weeks to mature so today you will observe a series of columns at different stages of maturity that have been prepared earlier in the semester for you. Using these columns as a guide, along with the materials in your tutorial, you'll discuss some of processes occurring in the column.

In our Winogradsky columns, sulphates are being reduced by microbial respiration into sulphides – a processes that involves the transfer of electrons. Electron transfer during microbial respiration can be thought of like an electrical current – electrons are moving from one community to another within the column. In fact, there is so much electron transfer occurring during microbial respiration that we can siphon electrons from the community and use the electrical current to power an LED light. We term this type of "battery" a Microbial Fuel Cell. In the second part of this practical you will use the microbial communities present in soil to make a Microbial Fuel Cell and measure the power output over the next 2 weeks.

This week you also receive the results from your high-throughput sequencing of 16S (SSU) rRNA sequences of your sample. Take some time to discuss with your mini-group and tutor what these results mean and how your pet microbe might fit with these results. For those that find themselves a little ahead of the class you might want to try the optional 'stretch goal' of doing a more in-depth analysis of your Illumina data using the software that we use for research in microbial ecology.

LEARNING OBJECTIVES

- Understand how nutrients and energy are passed between communities within the Winogradsky column.
- Understand how microbial respiration can be harnessed to convert microbial metabolism of a wide range of substrates into electrical energy.
- Be able to calculate cell density of your viable count plates

ACTIVITIES

1. Winogradsky column.
2. Soil based microbial fuel cells.
3. Maintenance of project microbes and ongoing phenotypic testing.
4. Discuss your high-throughput sequencing results within group.

Contents:

WEEK 4 RISK ASSESSMENT 75
WEEK 4 LABORATORY CLASS 76
1. Winogradsky column 76
Development of the microbial population in the column 77
1.1 Winogradsky column demonstration 79
2. Soil based microbial fuel cells 82
Materials 83
Procedure 83
3. Maintenance of project microbes 84
4. High-throughput 16S (SSU) rRNA sequencing data 84
5. Viable counts of microorganisms (continued) 78

WEEK 4 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk7

Hazards	Risks	Controls
General	See below	**At all times** follow demonstrators' instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Collecting biological/ environmental samples	Inhalation of bioaerosol particles (microorganisms) leading to inflammation, allergy, or to serious systemic infection by secondary pathogens.	PPE (lab coats, closed in shoes & gloves as required). Work in PC2 laboratory or Biological Safety Cabinet (BSC) if required e.g. for respiratory pathogens.
Sharps when assembling Mudwatt cells	Sharp wire injury	Take care when handling wire and place any unused sharp items in the sharps containers provided.
Handling hot items Electrical Equipment (water baths)	Electric shock/ electrocution burns	Ensure you are using heat protective mitts when handling hot objects. Stand to side of the water bath when opening it to allow steam to dissipate before removing items. Avoid water/spillages when working with electrical items.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 4 LABORATORY CLASS

1. Winogradsky column

You have reviewed the Winogradsky column in this week's tutorial, but if you'd like some more information before answering the questions below, here's a little refresher:

The study of microbial ecology can be approached in 2 ways:

(i) By isolating, identifying, enumerating and studying the physiology of individual bacterial types of the ecosystem

(ii) By studying the activity (for example, chemical conversions) of the microbial population in the natural habitat.

One of the previous lab experiments, the exercise on isolation and identification of a given bacterial type, focuses on the former approach.

Bacteria and Archaea exhibit an astonishing metabolic diversity, which far exceeds that of the eukaryotes (animals, plants, fungi and other higher organisms). Bacteria and Archaea literally keep the biological world turning by recycling all the mineral elements necessary for life support. Two famous microbiologists pioneered the study of these processes, Sergius Winogradsky (1856-1953) and Martinus Willem Beijerinck (1851-1931). In contrast to the pure culture studies of other pioneer microbiologists such as Louis Pasteur and Robert Koch, Winogradsky and Beijerinck studied the relationships between different types of microorganisms within mixed communities.

A simple laboratory experiment – the Winogradsky column – illustrates how different microorganisms perform their interdependent roles. The activities of one organism enable another to grow, and vice-versa. These columns are complete, self-contained recycling systems, driven only by energy from light! The columns are straightforward to set up with a glass tube, about 30 cm tall and 5 cm diameter. Mud is collected from the bottom of a lake or river and supplemented with cellulose, calcium sulphate and calcium carbonate, then added to the lower one-third of the tube. The rest of the tube is filled with water from the lake or river, and the tube is capped and placed near a source of light.

The Winogradsky column is a classic demonstration of the metabolic diversity of Bacteria and Archaea, revealing how microorganisms occupy highly specific niches according to their environmental tolerances and their carbon and energy requirements. The column also enables us to see how mineral elements are cycled in natural environments.

All life on earth may be categorised in terms of the organism's carbon and energy source. Energy can be obtained from light reactions (phototrophs) or from chemical oxidations (of organic or inorganic substances) (chemotrophs). The carbon for cellular synthesis can be obtained from CO_2 (autotrophs) or from preformed organic compounds (heterotrophs). Combining these categories, we get the four basic life strategies: photoautotrophs (e.g. plants), chemoheterotrophs (e.g. animals, fungi), photoheterotrophs and chemoautotrophs. Only in the bacteria – and among the bacteria within a single Winogradsky column – do we find all four basic life strategies!

Development of the microbial population in the column:

All the organisms are initially present in low numbers, but when the tubes are incubated for 2 to 3 months the different types of microorganism proliferate and occupy distinct zones where the environmental conditions favour their specific activities.

The filter paper (cellulose) simulates organic material in the natural environment. The large amount of cellulose initially promotes rapid microbial growth that soon depletes the oxygen in the sediment and in the water column. Only the very top of the column remains aerated because oxygen diffuses very slowly through water.

Heterotrophic bacteria initially grow aerobically using the cellulose as an energy source, and as they do so they use up oxygen (which can diffuse in only slowly through the water column) thereby establishing anaerobic conditions. Anaerobic heterotrophs continue to grow slowly, producing a range of metabolic end products such as organic acids and alcohols, CH_4 and H_2. Excess acidity is neutralised by the $CaCO_3$ maintaining a near neutral pH.

The only organisms that can grow in anaerobic conditions are those that ferment organic matter and those that perform anaerobic respiration. Fermentation is a process in which organic compounds are degraded incompletely; for example, yeasts ferment sugars to alcohol. Anaerobic respiration is a process in which organic substrates are degraded completely to CO_2, but using a substance other than oxygen as the terminal electron acceptor. Some bacteria respire by using nitrate or sulphate ions, in the same way as we use oxygen as the terminal electron acceptor during respiration.

Some cellulose-degrading *Clostridium* species start to grow when the oxygen is depleted in the sediment. All *Clostridium* species are strictly anaerobic because their vegetative cells are killed by exposure to oxygen, but they can survive as endospores in aerobic conditions. They degrade the cellulose to glucose and then ferment the glucose to gain energy, producing a range of simple organic compounds (ethanol, acetic acid, succinic acid, etc.) as the fermentation end products.

The sulphur reducing bacteria such as *Desulfovibrio* can utilise the end products of fermentation by anaerobic respiration, using either sulphate or other partly oxidised forms of sulphur (e.g. thiosulphate) as the terminal electron acceptor, generating large amounts of H_2S by this process.

In our own aerobic respiration, we use O_2 and reduce it to H_2O. The H_2S will react with any iron in the sediment, producing black ferrous sulphide.
This is why lake sediments (and our household drains) are frequently black. However, some of the H_2S diffuses upwards into the water column, where it is utilised by other organisms.

The diffusion of H_2S from the sediment into the water column enables anaerobic photosynthetic bacteria to grow. They are seen usually as two narrow, brightly coloured bands immediately above the sediment – a zone of green sulphur bacteria then a zone of purple sulphur bacteria.

The green and purple sulphur bacteria gain energy from light reactions and produce their cellular materials from CO_2 in much the same way as plants do. However, there is one essential difference: they do not generate oxygen during photosynthesis because they do

not use water as the reductant; instead they use H_2S. The following simplified equations show the parallel.

$6\ CO_2 + 6\ H_20 \rightarrow C_6H_{12}O_6 + 6\ O_2$ (plant photosynthesis)

$6\ CO_2 + 6\ H_2S \rightarrow C_6H_{12}O_6 + 6\ S$ (bacterial anaerobic photosynthesis)

The purple sulphur bacteria typically have large cells and they deposit sulphur granules inside the cells. The green sulphur bacteria have smaller cells and typically deposit sulphur externally.

The sulphur (or sulphate formed from it) produced by the photosynthetic bacteria returns to the sediment where it can be recycled by *Desulfovibrio* - part of the sulphur cycle in natural waters.

Most of the water column above the photosynthetic bacteria is coloured bright red by a large population of purple non-sulphur bacteria. These include species of *Rhodopseudomonas*, *Rhodospirillum* and *Rhodomicrobium*.

These bacteria grow in anaerobic conditions, gaining their energy from light reactions but using organic acids as their carbon source for cellular synthesis. So, they are termed photoheterotrophs. The organic acids that they use are the fermentation products of other anaerobic bacteria (e.g. *Clostridium* species), but the purple non-sulphur bacteria are intolerant of high H_2S concentrations, so they occur above the zone where the green and purple sulphur bacteria are found.

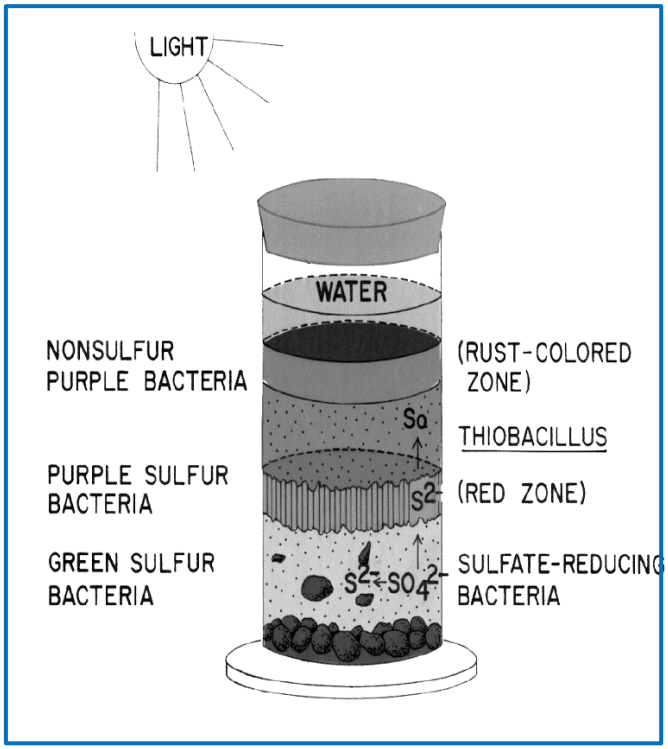
Many microorganisms can grow in the oxygenated zone at the top of the water column, but three distinctive types are of special interest.

(i) Any H_2S that diffuses into the aerobic zone can be oxidised to sulphate by the sulphur-oxidising bacteria. These bacteria gain energy from oxidation of H_2S, and they synthesise their own organic matter from CO_2. So they are termed chemosynthetic organisms, or chemoautotrophs.

Similar types of organism occur in soils, gaining energy from the oxidation of ammonium to nitrate, which then leaches from the soil and can accumulate in water supplies.

(ii) Photosynthetic cyanobacteria can grow in the upper zones. These are the only bacteria that have oxygen-evolving photosynthesis like that of plants. In fact, there is very strong evidence that the chloroplasts of plants originated as cyanobacteria (or the ancestors of present-day cyanobacteria) that lived as symbionts inside the cells of a primitive eukaryote. Similarly, there is equally strong evidence that the mitochondria of present-day eukaryotes were derived from purple bacteria. Once the cyanobacteria start to grow they can oxygenate much of the water.

(iii) The top of the water column can contain large populations of sheathed bacteria. These are aerobic organisms that use organic substrates but are unusual because as the bacterial cells divide they synthesise a rigid tubular sheath from which individual cells can escape and swim away to establish new colonies. Many empty sheaths are seen in older colonies. They are made of a complex mixture of protein, polysaccharide and lipid, and are thought to protect the cells from predation by protozoa. The sheaths also can be encrusted with ferric hydroxide, giving a yellow or rusty appearance to the colonies.



1.1 Winogradsky column demonstration

At the front of the class there are two mature Winogradsky columns for you to observe. When indicated by your tutor, head to the front of the class and observe the different stratified communities present in the column. Unlike most occasions in microbiology, you can see the different microbial communities and differentiate some simply by their colouring.

In the space overleaf, you should sketch the column and indicate the different communities that you can identify with your group and tutor. There are some specific questions below for you to answer back at your bench.

Sketch the Winogradsky column below and indicate and microbial communities that you can identify with your group and tutor.

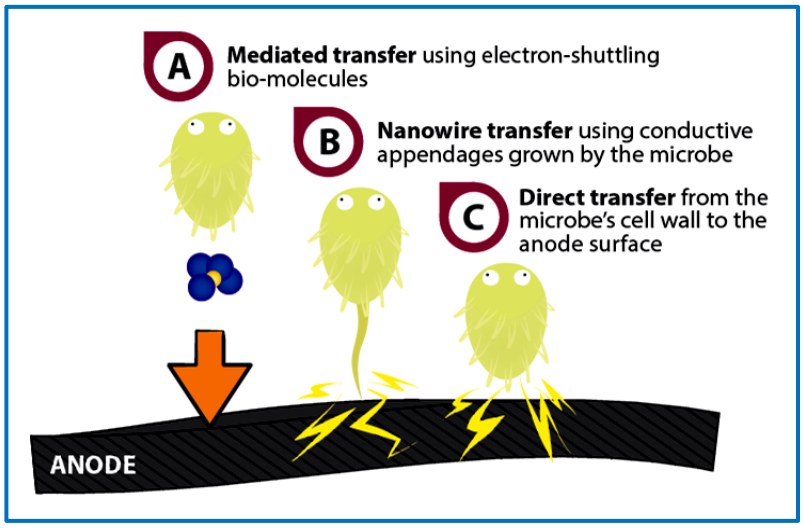
What is the purpose of the following ingredients that were added to the column?	
Mud	
Powdered cellulose	
Calcium sulphate	
Calcium carbonate	

Predict the location within the column and the chronological order of the appearance of the following:

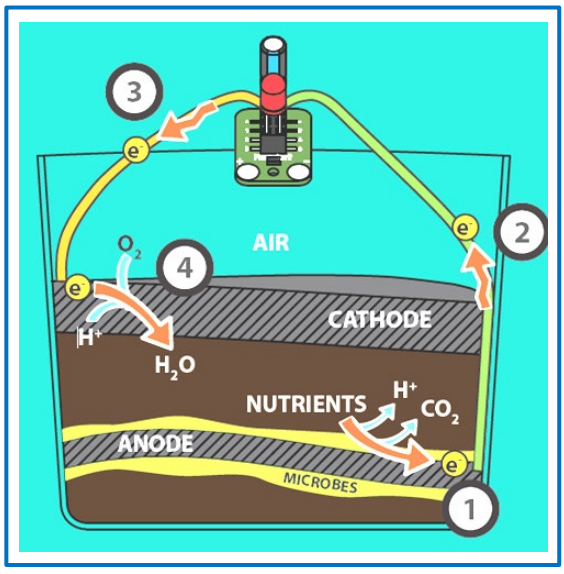
Organism	Reasons for location in column	Time of appearance
Anaerobic heterotrophs		
Green algae		
Sulphate-reducing bacteria		
Non-sulphur purple bacteria *(Rhodospirillum)*		
Aerobic heterotrophs		
Green sulphur bacteria (*Chlorobium*)		
Facultative anaerobic heterotrophs		
Cyanobacteria		
Purple sulphur bacteria *(Chromatium)*		

2. Soil based Microbial Fuel Cells

Harvesting energy from the environment is the primary activity of all living things. There are three principal ways to do this. Photosynthesis, fermentation and chemotrophic respiration. The latter is how we do it. As with humans, respiration in microbes involves the transfer of electrons to a suitable electron acceptor. For us this is oxygen, but for microbes there are many options. For example, bacteria can respire things like oxygen, nitrate, sulphate (seen in the Winogradsky column), iron, uranium, arsenic and even solid electrodes. Electrons can be transferred from bacterial cells to electrodes via soluble charge carrying molecules (electron shuttles), conductive appendages (nanowires or pili) or by direct cell contact.



The MudWatts provided (one per group) give us an opportunity not only to play with mud but also to bear witness to microbial respiration by linking the transfer of electrons from bacterial cells via an electrode to an LED. That's right, microbes can create enough potential in a bioelectrochemical system to make an LED blink. Not bad given the size of them. The following diagram illustrates the flow of electrons.



1. As the microbes around the anode munch up the nutrients in the mud, they **deposit electrons** onto the **anode**
2. These electrons travel through the wire to the **Blinker Board**, where they power the electronics.
3. The electrons then travel back down through the wire to the **cathode**.
4. At the cathode, electrons interact with oxygen and protons to form **water**.

This cycle happens over and over, trillions of times every second. This continuous flow of electrons is what we call electricity which can power small electronics.

Materials:

- Carbon felt cathode and orange wire
- Carbon felt anode and green wire
- Vessel, hacker board, capacitor and LED
- Additional items including, multimeters, buzzers and clocks will be available in ensuing weeks to play around with.
- Mud – this is 'sampled' from the beautiful gardens around UNSW… discreetly. You wil be provided will either:
    1. Soil
    2. Soil supplemented with extra organic carbon (cellulose)
    3. Soil that has been autoclaved and is now sterile

Procedure:

Completely saturate your soil with water.

Bend both wires 90° where the plastic sheath ends. Straighten out the bare end of the wire. The green wire will be used to make the anode, and the orange wire will be used to make the cathode.

Insert the bare end of the anode (green) wire into the side of the thin felt disc. Try to keep the wire from exiting the felt. Repeat this step with the cathode (orange) wire and the thick felt disc.

Pack a layer of mud into the bottom of your container, at least 1 cm deep. Pat down the soil so that you have a smooth layer.

Place the anode (with the green wire) on top of the layer of mud.

Fill your container with more mud, at least 5 cm deep, pressing down firmly to squeeze out air bubbles. Let your mud rest for a few minutes and drain any excess liquid.

Place the cathode (with the orange wire) gently on top of the mud. Do not cover the cathode with mud.
Attach the hacker board into the indentation on the lid.
Pass the electrode wires through the lid.

Facing the semicircular indentation, the cathode (orange) should be on the left and the anode (green) on the right. Now press the lid down onto the jar to snap it into place.

Connect the cathode wire (orange) to the '+' and connect the anode wire (green) to the '-'.

Place the long end of the capacitor to pin 1 and the short end to pin 2. Connect the long end of the LED to pin 5 and the short end to pin 6.

It will take a few days for the microbes in the soil to colonise the anode at the bottom of the vessel and for the LED to start blinking. Over the next 2 weeks you will record the electrical current produced by your Microbial Fuel Cell and then compare with groups using soil, soil + cellulose, or sterile soil. The Fuel Cells will be displayed in the Lab window and mutlimeters will be contected. You must organise for someone from your

group to take every 2-3 days. Record your results in the spreadsheet provided on the class OneNote Notebook (Content Library > Student Results > Soil microbial fuel cell results) and linked here.

3. Maintenance of project microbes

For those of you that haven't commenced or completed your phenotypic testing spend some time performing these tests as described previously. Don't forget to subculture your culture for future use. Photograph your culture plate and any of the tests you have performed. Keep your data and notes here.

Suggestive learning activity:

Weekly group discussion for your course project - check Collaboration Checklist for suggestive topics

Don't forget to record group meeting minutes (meeting minute template) and develop weekly action plan (And stick to the plan!).

4. High-throughput 16S (SSU) rRNA sequencing data

This week you'll receive the analysed data from the Illumina (high-throughput) sequencing of your DNA samples. It's important to realise that we haven't sequenced all of the genomic DNA in your sample, instead the 16S (SSU) rRNA sequences have been amplified from your sample DNA and this has been sequenced. This allows sensitive detection of the microbial species in your sample and is analysed using a conceptually similar pipeline to the one that you used last week. The sequencing data has been analysed for you at the Ramaciotti Centre for Genomics and you will be provided with an analysis of community composition to ponder and include in your final report. Discuss the results with your group and tutor.

5. Viable counts of microorganisms (continued)

Count the number of *S. cerevisiae* colonies on the three agar plates you prepared last week. Record your data:

Number of colonies on lowest dilution (least dilute):

Number of colonies on intermediate dilution:

Number of colonies on highest dilution (most dilute):

Cell concentration in original solution (CFU/mL) =

Was the 10-fold dilution series apparent in your colony counts? If not, give reasons why:

END OF WEEK 4

OVERVIEW

This week you'll be checking on your pet microbes to see if you've isolated pure cultures and making your first steps into the exciting world of microbial DNA sequence analysis.

LEARNING OBJECTIVES

- Be able to generate a phylogenetic tree from bacterial 16S rRNA sequences.

ACTIVITIES

1. Course project: Sub-culturing and storing project microbes
2. Introduction to sequence analysis
3. Record the results of your Microbial Fuel Cell

Contents:

WEEK 5 RISK ASSESSMENT 87
WEEK 5 LABORATORY CLASS 88
1. Course project: sub-culturing and storing project microbes 88
Procedure 88
2. Introduction to sequence analysis 90
Exercise 1. Accessing software for phylogenetic analysis of SSU rRNA sequences 93
Exercise 2. Sequence browsing and alignment of SSU rRNA gene sequences 94
Opening an Alignment 95
Aligning Sequences Using Muscle 96
Quality control of your alignment 96
Exercise 3. Estimating evolutionary distance and creating phylogenetic trees 100
Build a phylogenetic tree from your evolutionary distance matrix 101
Exercise 4. BLAST search for closest relatives using the NCBI database 102
3. Record the results from your Microbial Fuel Cell 98

WEEK 5 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_COMP

Workstation set-up		
Ergonomics Electrical	Musculoskeletal pain. Electrical shock/fire	Correct workstation set-up. Check electrical equipment in good condition before use. All portable electrical equipment tested and tagged.

Elbow at 90º angle

Adjust seat back for lumbar support

Monitor tilt

Personal Protective Equipment
Not necessary in these practicals. Ensure laboratory has been set up as a DRY laboratory (see door signage). Use benchcoat under laptop/tablet if required.

Emergency Procedures
In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal
No apparatus used in these practicals.

Declaration – will be checked by demonstrator at the beginning of the practical class
I have read and understand the safety requirements for this practical class and I will observe these requirements. Signature:……..……Date:…… Student number:……..

WEEK 5 LABORATORY CLASS

1. Course project: sub-culturing, MALDI-TOF and storing project microbes

Examine your agar plates from last week. Take a photo and save to a location where you can access it for your final report (create a MICR2011_Report folder in your student drive). Based on colony morphology do you have a pure culture or mixed lineage growth on your plate? Pick a colony and subculture onto a new plate. Check next week that your microorganism is still alive. To ensure the culture is not lost during the following weeks stocks of the culture can be frozen and revived at a later time point if necessary as follows.

Procedure:

- Only continue today with the following if you are sure you have a pure culture. Otherwise, this step can be performed next week.
- Obtain a culture plate showing good growth of a pure culture of your isolate.
- Aliquot 0.5 mL of Luria broth (containing 15% glycerol) into a cryotube.
- Using a sterile loop, pick two or three colonies and transfer and suspend in the cryotube from above.
- Labeled with your name and date. Give this to your demonstrator who will store it at -80˚C until required.

Identify your microorganism

Once you are satisfied that you have a pure culture, you may begin your identification. You will use two methods: a more classical biochemical characterisation, and phylogenetic analysis of the 16S rRNA gene from your microorganism.

Flow charts of the tests required to identify your microorganism are provided in Appendix 1 and are adapted from Bergey's Manual of Determinative Bacteriology. The stains and tests listed (and instructions for performing the tests) are available in the lab and you should discuss with your tutor the best way to identify your microorganism. You will need to start with a Gram stain to determine the Gram reaction and cell morphology. This will also help you determine if you have a pure culture.

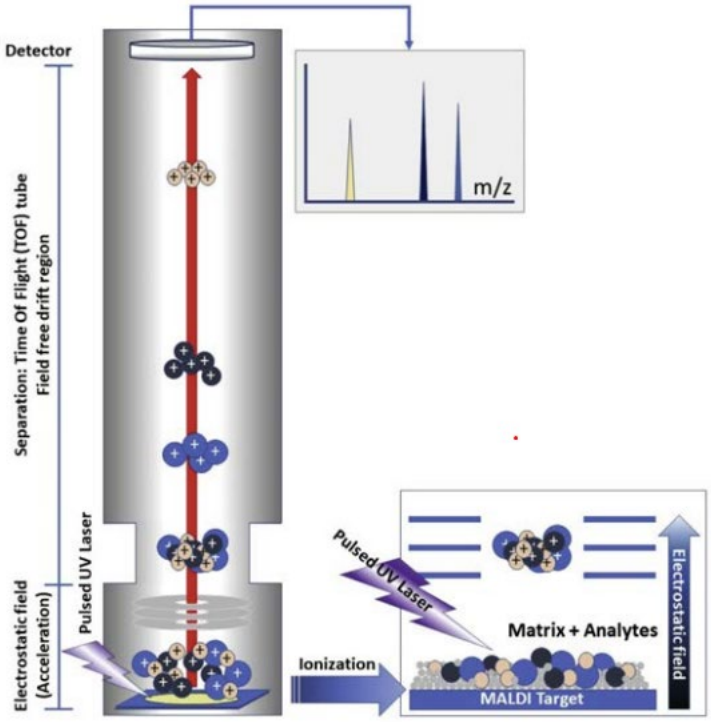
In the next practical, you will sequence part of the 16S rRNA gene from your microorganism and, using the skills you will learn today, identify your microorganism using phylogenetics.

MALDI-TOF mass spectrometry analysis of project isolates

Mass spectrometry (MS) is a relatively recent addition to the microbiologist's toolkit. MS can be used to create a "fingerprint" of the molecular weight of the most abundant proteins in the cell. These proteins are predominantly ribosomal proteins and there is sufficient variation in the molecular weight between genus, species, and sometimes strains to determine the microbe's identity.

The major advantage of using MS to identify microorganisms is the speed of identification. From a pure culture, you can identify the microorganism in less than an hour. Almost all MS instruments for bacterial identification use matrix-assisted laser desorption/ionization (MALDI) to vaporise and ionize your sample microorganism for MS. Your sample is mixed with a “matrix” solution, in this case we will use α-Cyano-4-hydroxycinnamic acid (HCCA) that will absorb energy from the UV laser of the MALDI to generate free radicals and heat that will ionize and vaporise the sample.

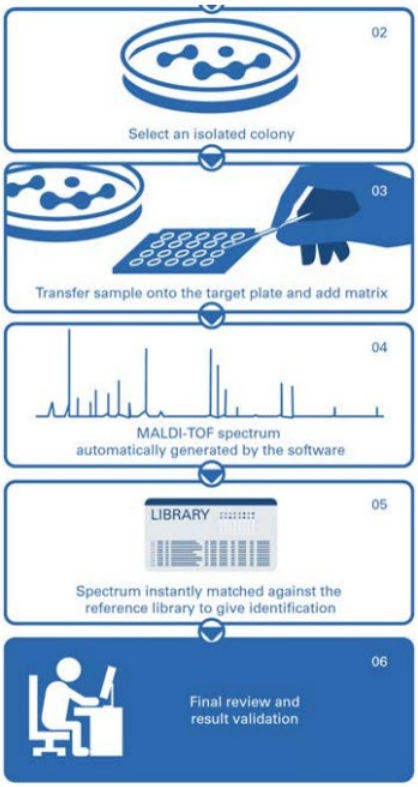
The ionised sample vapour containing proteins from your bacterial sample is then fed into a Time-Of-Flight (TOF) mass spectrometer, hence the name MALDI-TOF. The TOF spectrometer will measure the mass/charge ratio (m/z) of the proteins in the sample creating a fingerprint of the molecular weights of the most abundant proteins in the cell. To identify our microorganism we compare the spectra from the 2-20 kDa range of proteins against a database of spectra from previously characterised bacteria. In this way we can match the MALDI-TOF fingerprint of your unknown microorganism to known samples.



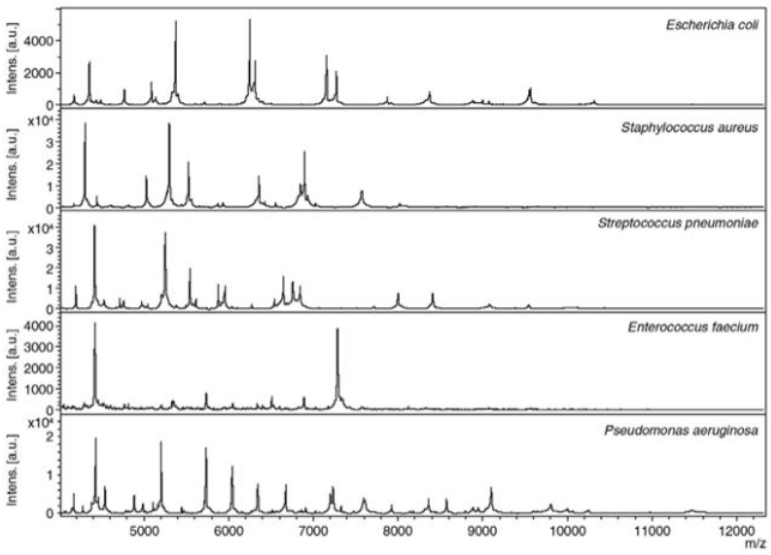
Schematic representation of MALDI-TOF instrument (Hou *et al* Journal of Food and Drug Analysis 2019).

We will use the MALDI-TOF Biotyper located in the Bioanalytic Mass Spectrometry Facility (BMSF) here at UNSW. They will add matrix (HCCA) to your sample, process it in the MALDI-TOF Biotyper, and provide you with database of matches for your sample.

Today, you just need to streak out a colony from you pure culture for your demonstrator. Ensure that it clearly labelled with your group number, name, and practical class.



Workflow used to analyse samples with the Bruker MALDI-TOF Biotyper.



MALDI-TOF spectra used to fingerprint five bacterial isolates (indicated right) (Carbonelle *et al* Clinical Biochemistry 2011)

2. Introduction to sequence analysis

Bioinformatics now plays a central role in medical and environmental microbiology. From tracking the evolution of pathogenic viruses, to identifying a fungal isolate, or identifying bacteria in a sputum sample, making sense of DNA sequence data is crucial.

Over the past decade there has been a technological revolution in sequencing, with the costs dropping dramatically and the amount of sequence data increasing. For example in 2001 the cost of the Human Genome project was $100 million. Today genomes can be sequenced for around $1000.

This revolution has meant that sequence data is much more readily available to a wide range of scientists than was previously the case. In microbiology, this has resulted in the capacity to deeply sequence the DNA of microbes from any sample and readily identify community composition and function. As a consequence, much of the skill involved in medical and biotechnological research has moved from the “wet lab” to the computer lab, and you will need to become comfortable using (or even developing) many types of bioinformatic software during your career.

One good thing about playing around with bioinformatics is that you can’t really do any harm, spread a dangerous virus, burn down the lab, etc. You can probably take down a server with some bad code but this can generally be fixed pretty easily. Feel free to explore the software we will use (and any other similar packages you can find). Much newly developed software is open source, so if you are computationally inclined downloading, installing and trying out new methods is a great way of advancing your

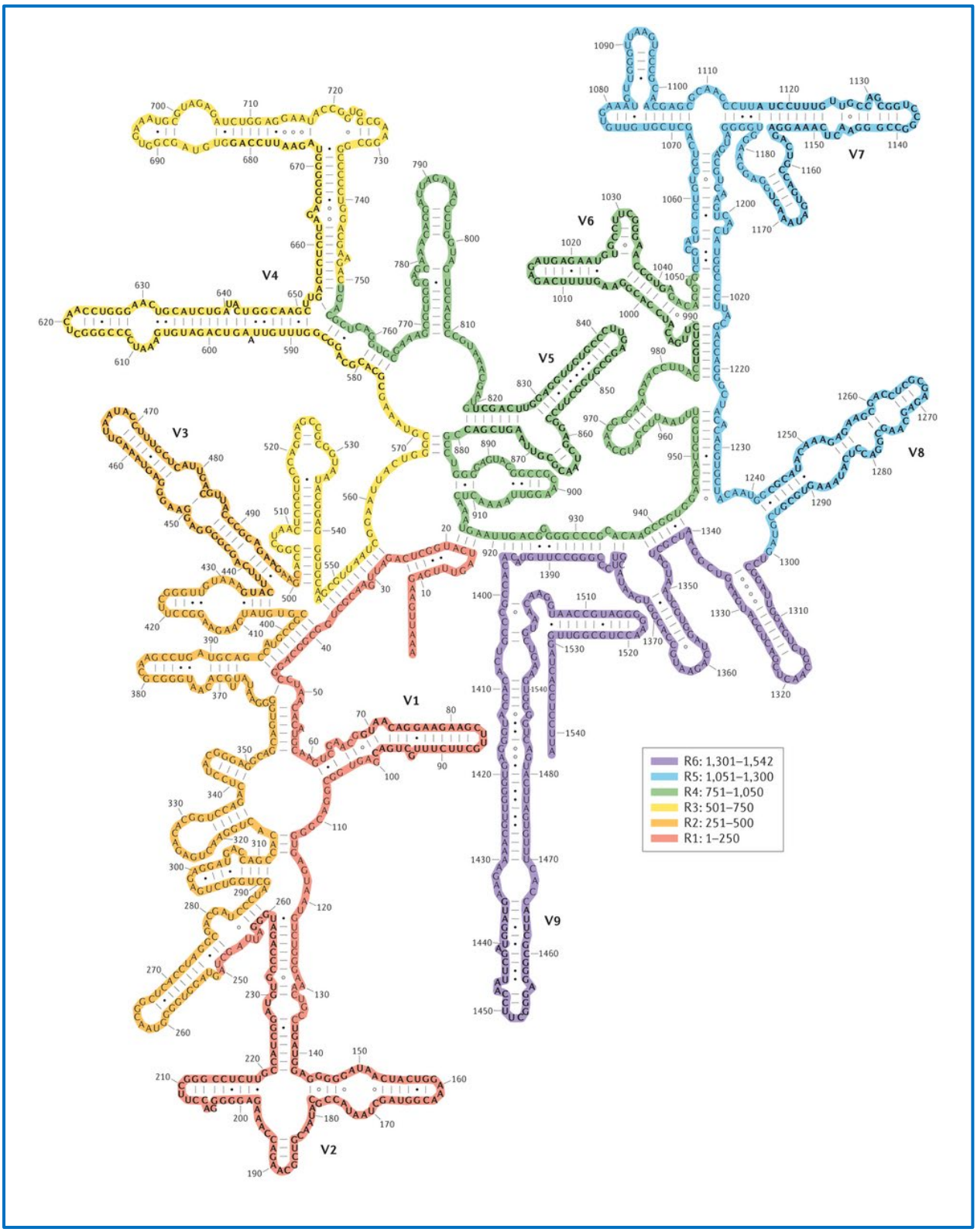
knowledge. If you are less into computers then there are still many ways to get the information you need out of sequence data and move on.

There are many different ways to do most analyses, and as long as you develop the conceptual knowledge of what you do at each step of the process you have the flexibility to move between different tools as you see fit. Understanding and flexibility ensure you'll be able to deal with things when they don't go as expected and build checks into your workflows to ensure things stay on track.

We will use both old and new sequencing methods in this course. The newer methods are currently known as “next-generation” sequencing technologies. These are for sequencing complex samples and will be discussed next week. The “old-school” method of sequencing is called Sanger sequencing, which is typically used to identify an isolated microbe. It is named after its inventor Fredrick Sanger, who won two Nobel prizes in chemistry (he is one of only two people to have won two Nobel prizes in the same category – who is the other one?).

One of his prizes was for developing a robust method for sequencing DNA as you saw in this week’s tutorial. Later in the course you will use Sanger sequencing to identify your project microbe.

Today we are going to get a feel for what sequence data looks like and how comparing sequences works. We will look at small subunit (SSU) rRNA gene sequences. These are transcribed to RNA (but not translated to proteins). The SSU rRNA molecule (shown below) becomes part of the structure of the ribosome – which every cellular organism on earth needs to make proteins. Hence these genes are very important, very conserved, and present in every organism, so we can compare them to quantify similarity and build phylogenetic trees showing relatedness between organisms.


V1
V2
V3
V4
V5
V6
V7
V8
V9
R6: 1,301–1,542
R5: 1,051–1,300
R4: 751–1,050
R3: 501–750
R2: 251–500
R1: 1–250

The secondary structure of the SSU rRNA molecule is useful to understand the methods we use to explore diversity using the gene. When folded in two dimensions RNA forms a series of stems and loops. In general, as the stems require base-pair bonding, it is important that these regions of the gene are conserved. Nucleotide mutations in stem regions can stop secondary structure formation and render this essential molecule useless and the organism will die. That's a strong selection pressure for conservation. Loop sequences on the other hand don't base pair so they mutate more and are less conserved.

When you learn and read about using Illumina sequencing to examine microbial assemblages from different environments you will hear about variable regions V2-V3, or V4, or V6. These designations refer to specific sub-parts of the SSU gene that contain variability between species so are useful for telling one species from another.

Exercise 1. Accessing software for phylogenetic analysis of SSU rRNA sequences

Today you will be working in pairs and using a software package called MEGA for analysis of your mock SSU rRNA sequences. You will use MEGA to analyse your 16S rDNA Sanger sequencing results of your pet microbe for your final report.

The MEGA software package is freeware that allows us to visualise and manipulate sequence data, create multiple alignments, estimate evolutionary distance, and build phylogenetic trees, amongst many other uses. You can access MEGA using your student zID through myAccess:

https://www.myaccess.unsw.edu.au

Click on `Access my apps` and select `MEGAX` from the arrayed App icons. If you are working on a personal computer you will need to install Citrux using the instructions on the myAccess page.

Once you have access to MEGAX you will need to download the file containing 16S rDNA sequences from the MICR2011 Moodle page under Week 5**.** Save the file on location that you can access at a later date, like your student drive.

Exercise 2. Sequence browsing and alignment of SSU rRNA gene sequences

The format of the file is called the FASTA format, a common sequence data format. FASTA files contain two text strings for each sequence – the first starts with a ">" and contains the name of the sequence. The second line contains the DNA sequence.

For example:

```
>Prochlorococcus_sp_MIT9313_gene_Sequence
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGACG
GGTGAGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGC
GGACGGGTGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAG
TGGCGGACGGGTG
```

Note that the DNA sequence is all one single text line but is written across multiple lines to fit neatly on this page.

A FASTA file can contain more than one sequence, for example:

```
>Prochlorococcus_sp_MIT9313_gene_Sequence
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGA
CGGGTGAGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAG
TGGCGGACGGGTGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCG
GGTTAGTGGCGGACGGGTG

>Prochlorococcus_sp_MIT9313_gene_Sequence
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGA
CTGGCGGCGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGACTGGCGGC
GTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGACTGGCGGCGTGCTTAA
CACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGA

>Escherichia_coli_st_DP17_gene_JF895181
TGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGG
CGTGCTTAACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGACTGGCGGCGTGCTTA
ACACATGCAAGTCGAACGAACCTTCGGGTTAGTGGCGGACTGGCGGCGTGCTTAACACATGC
AAGTCGAACGAACCTTCGGGTTAGTGGCGGA

>Escherichia_coli_st
AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGT
CGAACGGAAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACA
TGCAAGTCGAACGGAAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCC
TAACACATGCAAGTCGAACGGAAATTGAAG
```

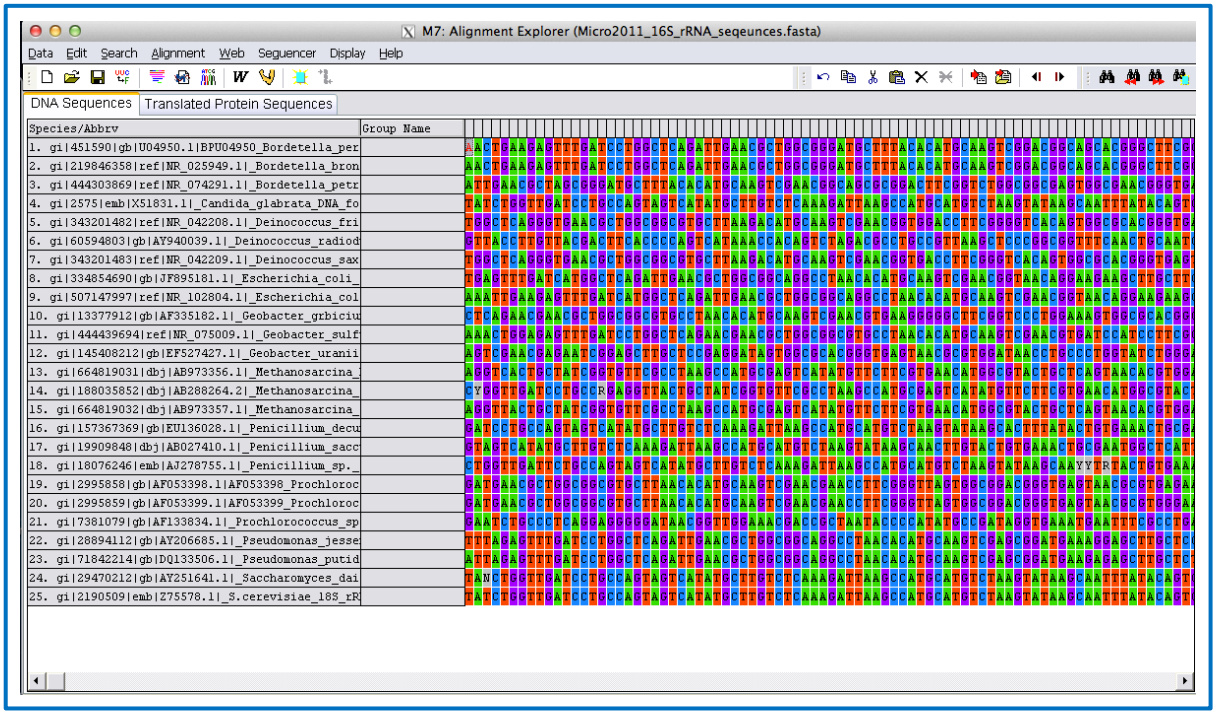
Handy hint – text editors like TextWrangler (OSX), NotePad (Windows), or vim/nano (Linux) are useful for opening and editing FASTA files. FASTA files can also be viewed using Microsoft Word but make sure you save in plain text format (.txt) as Word can insert additional hidden characters into your file. You can open the file named 'Week_5_16S_rDNA_sequences.fasta' in a text editor to check.

Opening an Alignment

The *Alignment Explorer* is the tool for building and editing multiple sequence alignments in *MEGA*.

1. Launch the *Alignment Explorer* by selecting the `Align` > `Edit/Build Alignment` on the launch bar of the main *MEGA* window.
2. Select `Create New Alignment` and click `OK`. A dialog box will appear asking, “Are you building a DNA or Protein sequence alignment?” Click the button labeled “`DNA`”.

3. From the *Alignment Explorer* main menu, select `Data` > `Open` > `Retrieve sequences from File`. Select the ‘Micro2011_16S_rRNA_sequences.fasta’ file from the location that it was saved. You should see something like this:



Notice the different coloured blocks for each nucleotide (A= green, T= red, C = blue, G = purple)

Use the scroll bar at the bottom to see how long each of the sequences are.

You can manipulate the order of the sequences by dragging and dropping. Try moving the sequences up or down in order by dragging the names.

For example, move Candida_glabrata down so it is above the two Saccharomyces sequences at the bottom. Then move all the eukaryotes to the bottom (Saccharomyces, Candida and Penicillium)
Double click in the “Group Name” box next to one of the sequences and type in something to represent the Genus name. e.g. ‘Pro’ for *Prochlorococcus*. Do this for the rest of the groups (e.g. ‘Bord’ for the *Bordetella* sequences). Group *Candida* with the *Saccharomyces*.

Aligning Sequences Using Muscle

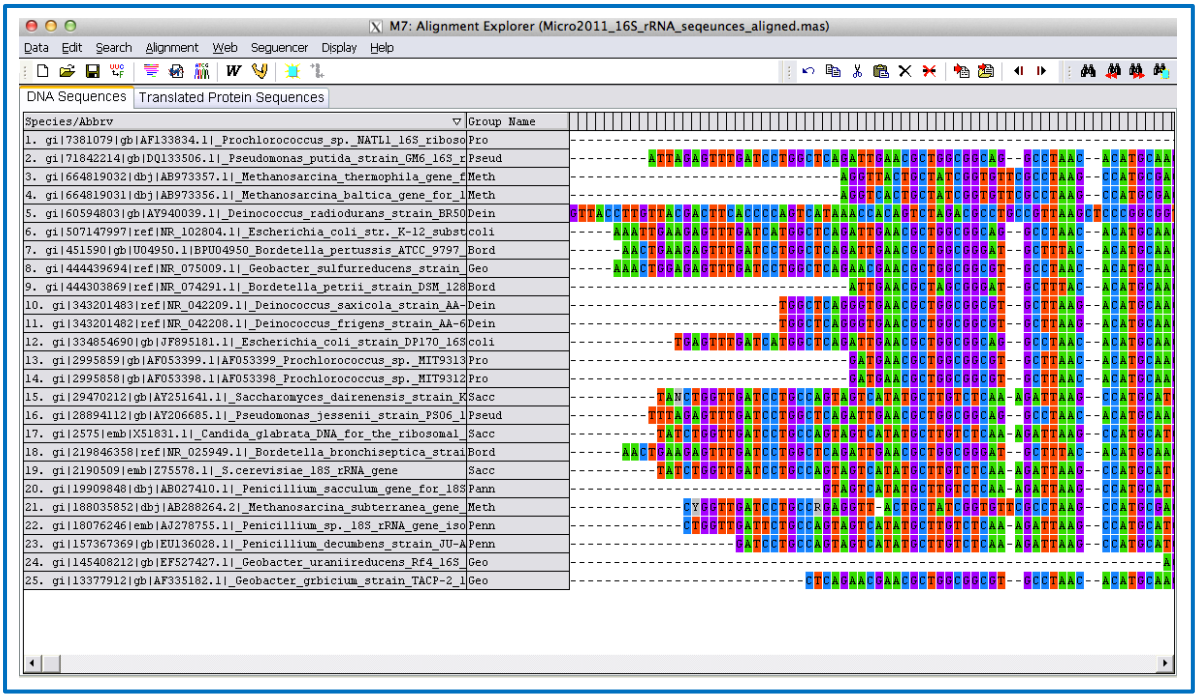
Before you can do ANYTHING with your sequences they must be aligned (matched up so that you compare the right base pairs to each other).

1. On the *Alignment Explorer* main menu, select `Edit` > `Select All`.

2. Select `Alignment` > `Align by Muscle` from the main menu to align the selected sequences data using the Muscle algorithm. Near the bottom of the *MUSCLE - AppLink* window, you will see a row called *Alignment Info*. You can read information about the Muscle program.

3. Click on the `Compute` button (accept the default settings). A *Progress* window will keep you informed of Muscle alignment status.

When the Muscle program has finished, the aligned sequences will be passed back to *MEGA* and displayed in the *Alignment Explorer* window. When its finished you should see something like this, with the sequences moved in the alignment so they "match" up correctly.

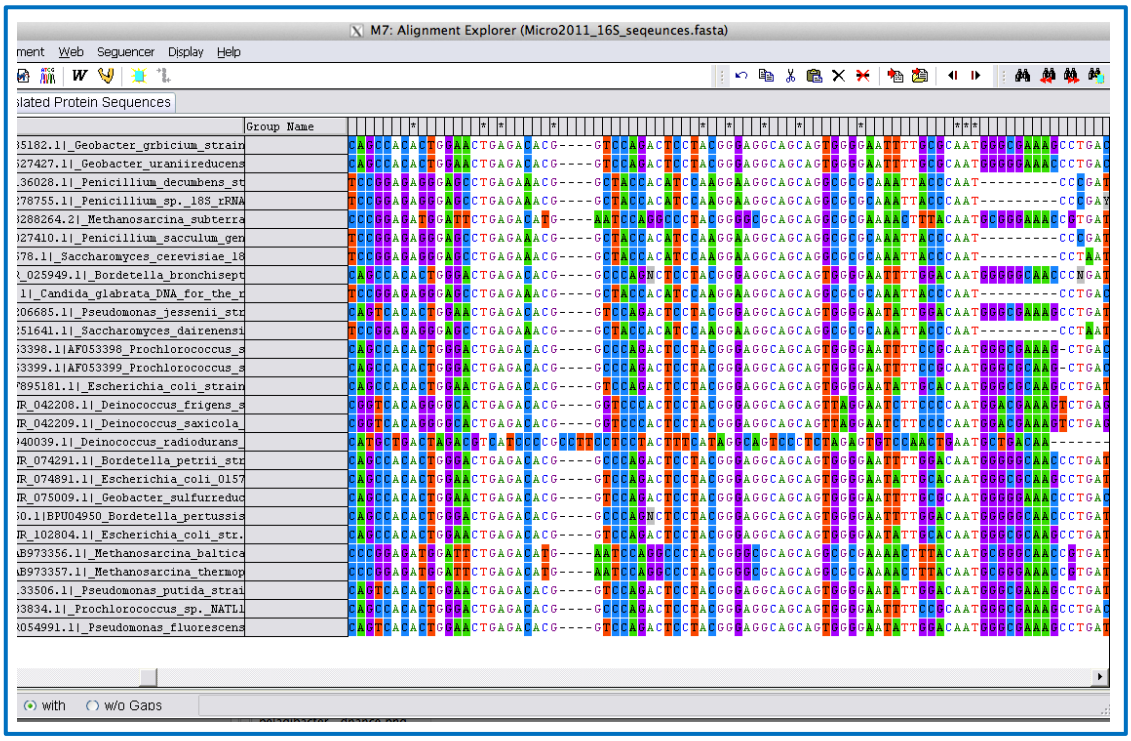


Now scroll around and have a look at the alignment. Look for conserved regions and hyper-variable regions. To help look for conserved regions (where all the species have the same base pairs) go to `Display` > `Toggle Conserved Sites` and set this to 80% - this means that whenever 80% of the sequences have the same base pair in one position that position is considered conserved.

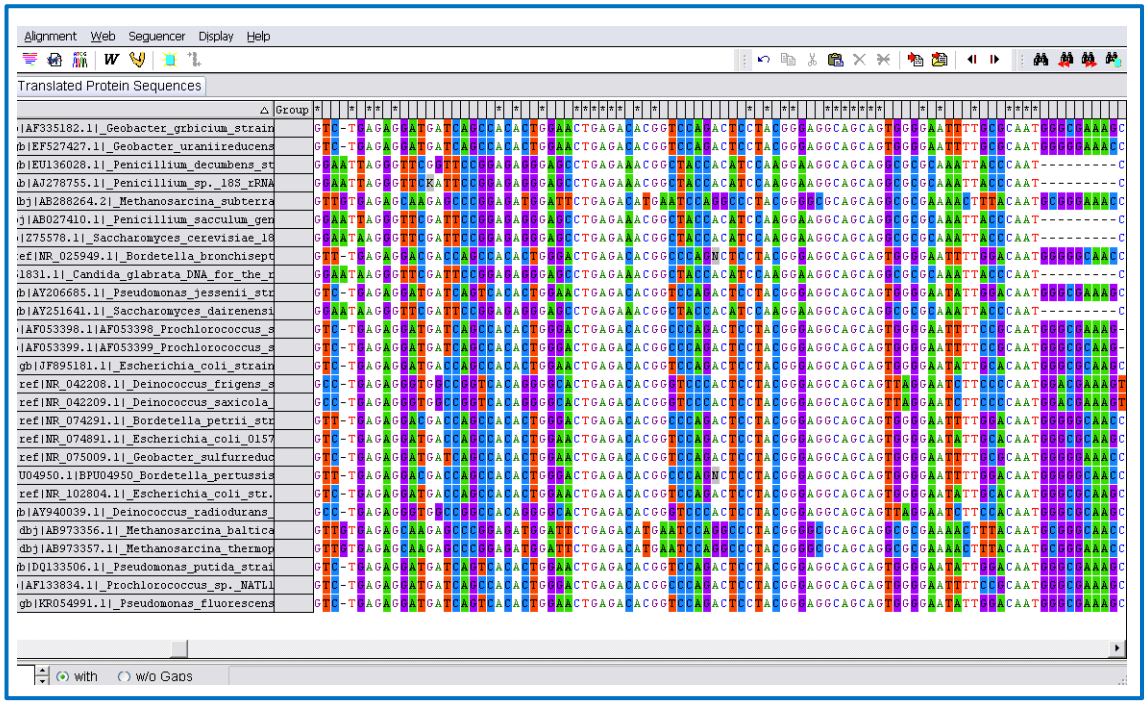
Quality control of your alignment

Doing this highlights the conserved and variable regions better. Notice how *Deinococcus radiodurans* (AY940039) looks much different to all the other sequences? Even the other *Deinococcus* sequences. This suggests that we have imported the sequence in the wrong

orientation, or reverse complemented. (Mostly sequences are read in the 5' to 3' orientation, but occasionally someone will submit a sequence to a database in the 3' to 5' orientation, and it's hard to tell without comparing it to other sequences.)



To fix this click on the name of AY940039.1|_*Deinococcus radiodurans* sequence to highlight it, then go to `Data > Reverse complement`. This will change the orientation of this sequence. Now you need to select all the sequences and realign them, as above (steps 1-3). Now the alignment of this sequence should look better.



Now we want to make a phylogenetic tree with our sequences to see how they are related. To do this we need to compare the same REGION of the SSU rRNA gene across all species. Notice how the *Prochlorococcus* NATL1 sequence is shorter than most of the others, both

at the start and at the finish. This is a problem because you can't accurately compare sequences of different length. To resolve this we need to do one of two things:

- Delete the base pairs of all the other sequences from the start and end so they match up with NATL1. This will allow us to keep NATL1 in our tree but remove a lot of data we use to build the tree, or

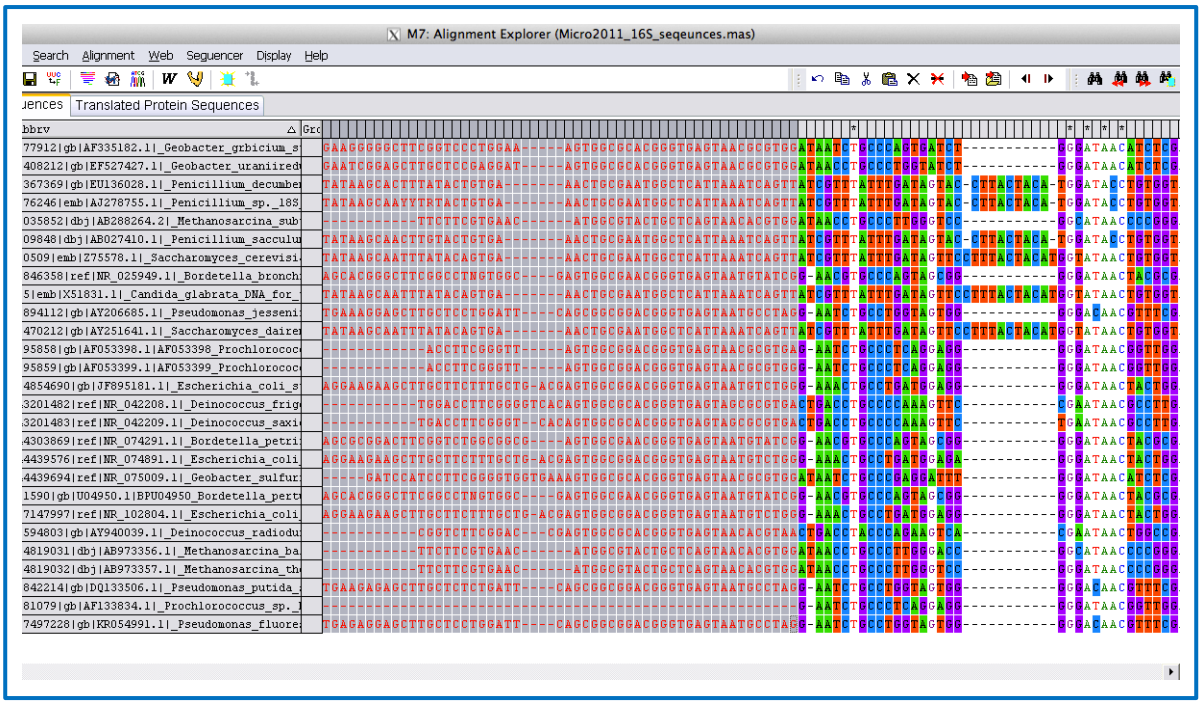
- Remove NATL1 from the alignment before we make the tree, and just trim the ends to make the other sequences the same length.

Your decision will depend on whether you are interested in the position of NATL1 and the intergroup similarities in *Prochlorococcus*, or more so in the rest of the sequences?

If you want to remove NATL1 click on the name and then `Edit` > `Delete` to remove it.

OR

If you want to keep all your species for now, delete all the columns in the alignment from the start of the alignment to the start of the NATL1 sequence, and from the end of the alignment to the end of the NATL1 sequence, by highlighting the appropriate grey boxes at the top of the alignment and then `Edit` > `Delete`.



Note that if you got rid of NATL1 you still need to trim the remaining sequences to make sure all sequences are the same length. Once our alignment is in order we are ready to compute evolutionary distances between species.

- Save the alignment file: `Data` > `Export alignment` > `MEGA Format` using the prefix `Micro16S_aligned_` with the date and your name. For example: Micro16S_aligned_20180328_Carl_Woese

Consider the following questions:

Next generation sequencing, such as Illumina sequencing, is a common method to survey community composition in microbial ecology. Because Illumina sequences are only short, the entire length of the SSU rRNA gene cannot be sequenced.

Which regions would be good for a PCR primer set aimed at amplifying both Bacteria, Archaea AND Fungi in one assay?

Which regions would be good to analyse just the Bacteria?

Which regions would be good for a quantitative PCR assay targeting the Prochlorococcus species?

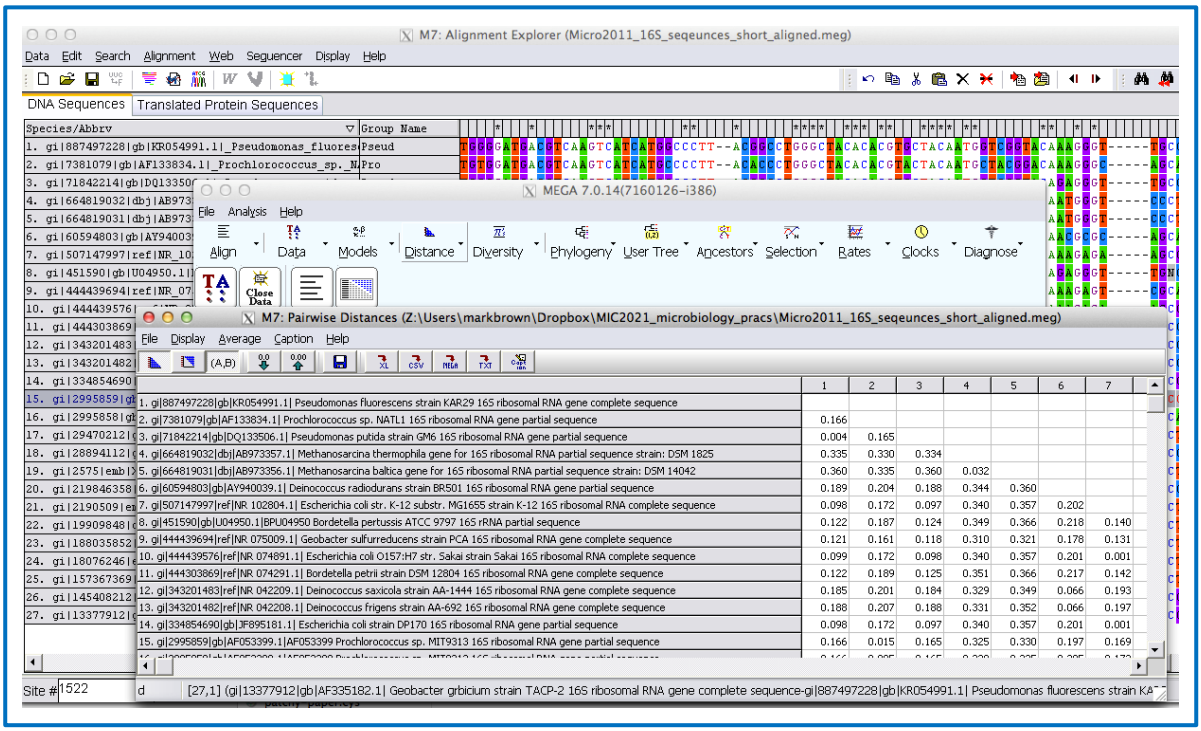
Exercise 3. Estimating evolutionary distance and creating phylogenetic trees:

1. From the main MEGA launch window, select `Distance` > `Compute Pairwise Distance`.

2. Choose to use the alignment file you just saved.

3. Click `OK` to begin the computation.

A progress indicator will appear briefly and then the distance computation results will be displayed in grid form in a new window. Leave this window open so we can compare the results from the next steps.



This type of grid is a lower triangle. The first column of numbers indicates the pairwise alignment value of each sequence to the first sequence in the list (0.000 means they are identical, 1.000 means they share no base pairs in common) (e.g. *Pseudomonas fluorescens* is 0.166 (16.6%) different to *Prochlorococcus* NATL1 (or they are 83.4% similar) but only 0.004 different (99.6% similar to *Pseudomonas putida*). The second column indicates the pair-wise comparison between each sequence and the second sequence on the list. You can look at the average difference both within and between your groups by clicking on the Average tab.

Note: These are essentially the numbers that you would use to group your sequences into operational taxonomic units when using Illumina sequencing data. How different do you think these numbers would be if using only a small variable region as opposed to the full-length SSU gene? And how different would the results be comparing different variable regions?

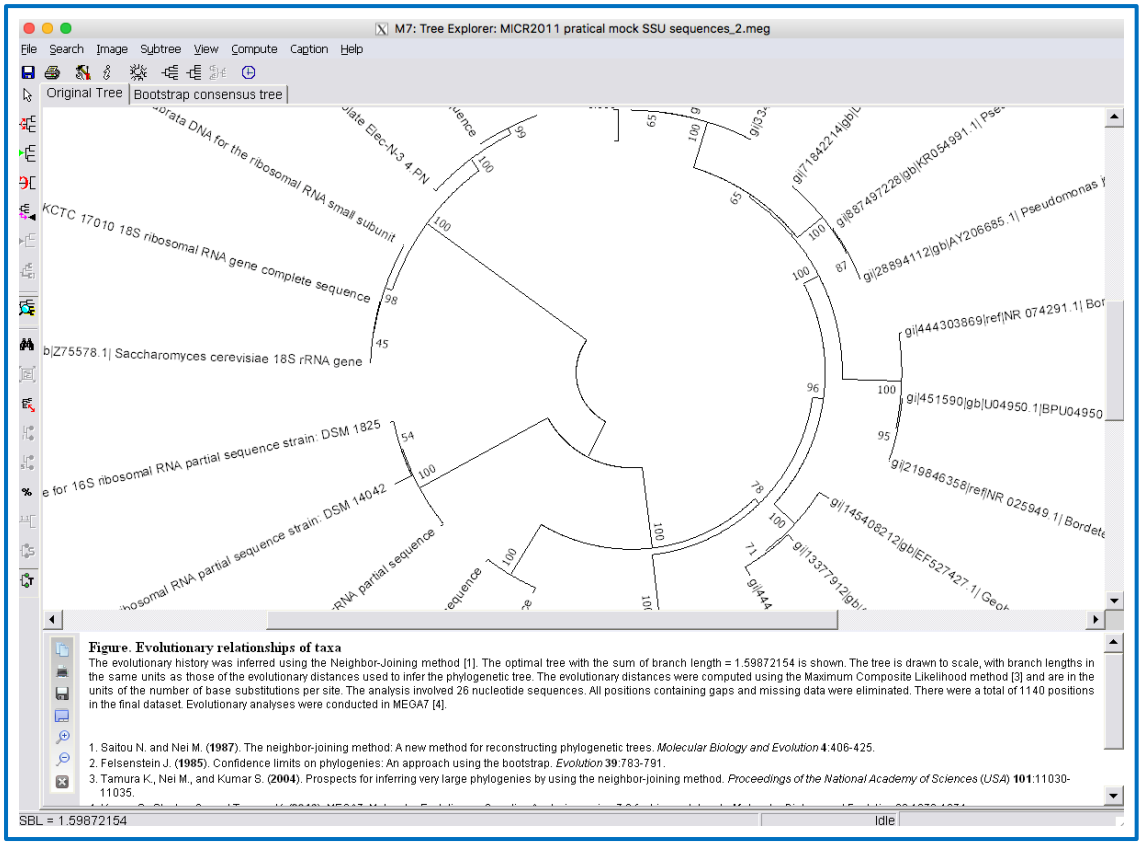
Build a phylogenetic tree from your evolutionary distance matrix

1. Go back to the main MEGA window and click on `Phylogeny` > `Construct/Test Neighbour-Joining tree`.

2. In the `Analysis Preference` window, click `Test of Phylogeny` and select `Bootstrap method`. Set the `No. of Bootstrap Replications` to 100. This is actually quite a low number and ideally you would use 500-1000 replications – but we don't have time to wait for it to process today, so 100 will have to do. You can use a 500-1000 replicates for your Sanger sequences in Week 11. Bootstrapping is a method to determine the confidence that each branching point in your phylogenetic tree is true. In essence, the tree will be regenerated (replicated) the specified number of times, and to determine how robust each branch point is, in each replication a few nucleotides will be replaced. The bootstrap value represents how many of the new phylogenetic trees contain the branch point. For instance, a bootstrap value of 95 indicates that 95/100 phylogenetic trees generated contained that branch point. A bootstrap value of 95% confidence for a branch point is generally thought to be robust.

3. Click `Compute` to accept the defaults for the rest of the options and begin the computation. A progress indicator will appear briefly before the tree displays in the `Tree Explorer` window. This may take a few minutes to process with the bootstrap replication.

Some taxa may be hidden in this tree view as they are very closely related. Play around with the tree viewer and see what you can do, for instance try `View` > `Tree/Branch Style` > `Circle`.

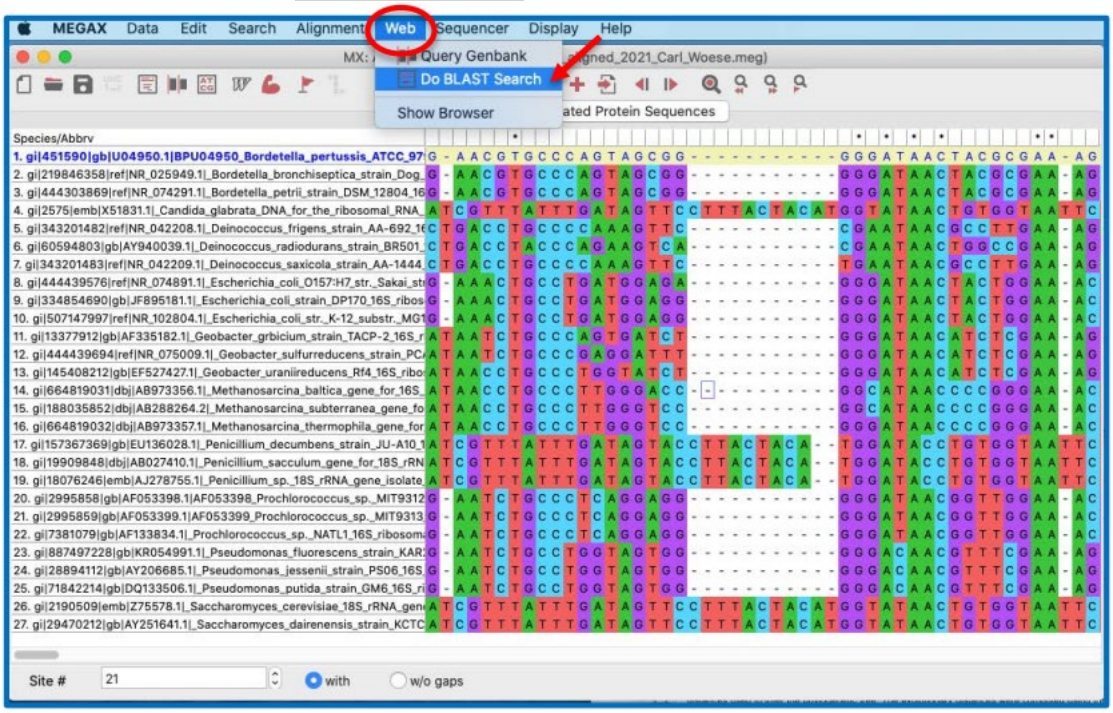


Think about how this process can help you identify an unknown sequence.

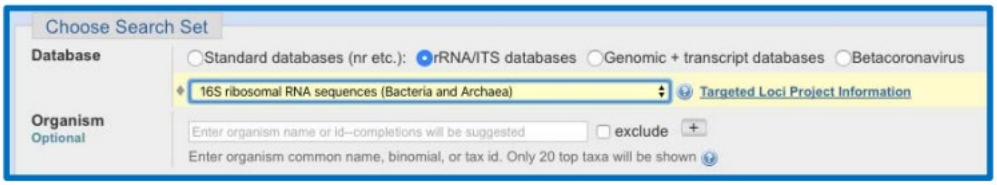
Exercise 4. BLAST search for closest relatives using the NCBI database:

The National Centre for Biotechnology Information (NCBI) is the major repository for all sequence data in the world. We can compare our sequences to this database to see if there are other sequences out there that are similar to ours.

For this activity you will need to navigate to the `Alignment Explorer` window (you may currently be working in the `Tree Explorer` window).

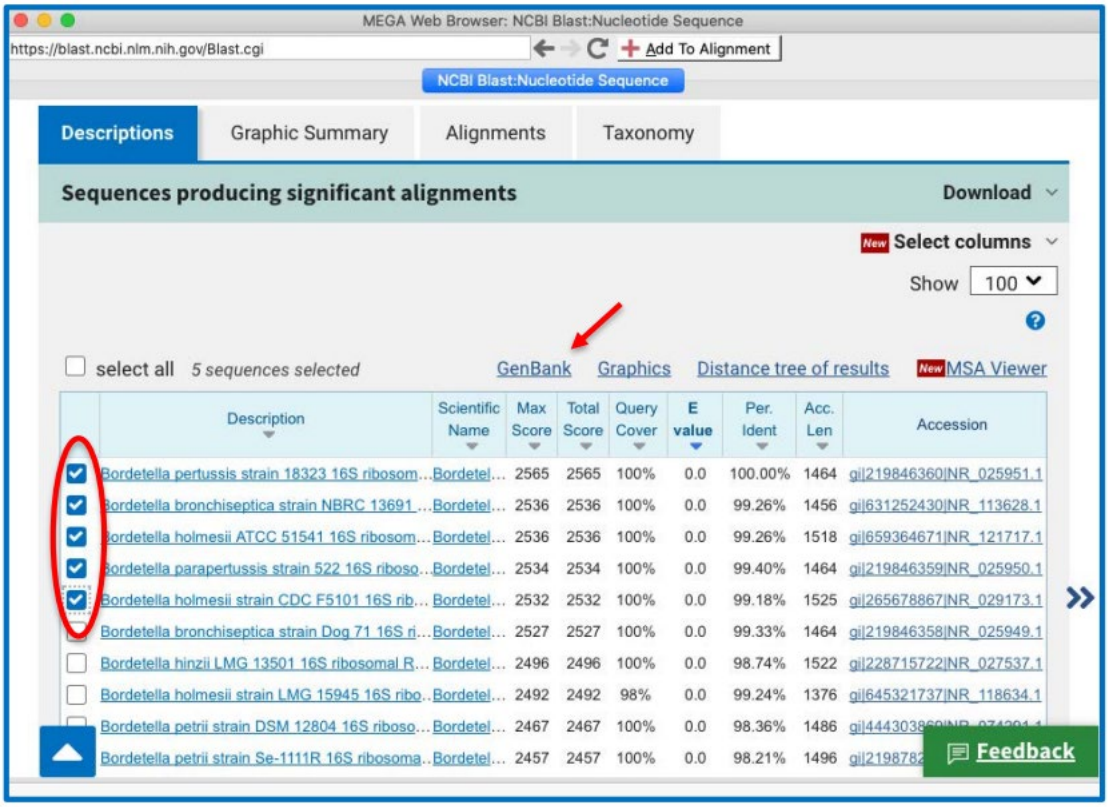


1. Click on the name of a sequence to highlight it, then click `Web` > `Do BLAST Search`. This will open the website of NCBI with your highlighted sequence ready to BLAST (align your sequence with the large database at NCBI). Make sure you select `16S ribosomal RNA sequences (Bacteria and Archaea)` from the database dropdown.

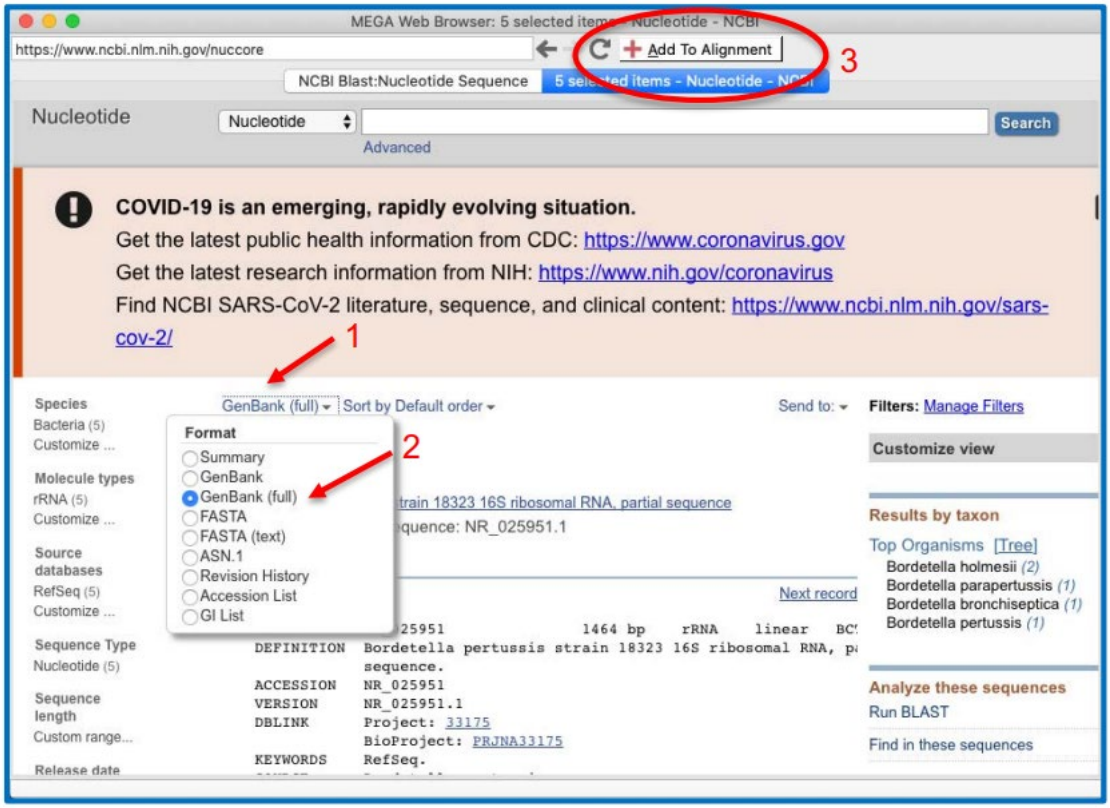


Scroll down and click.

2. This will retrieve 100 sequences most similar to yours in the NCBI database. If you had an unknown sequence you could BLAST it first, download the similar sequences and add them to your analysis. You will do this in Week 11 after we sequence your project microbes. Today we'll import the top 5 sequences – click the check boxes next to the BLAST hit, then click `GenBank` to obtain the correct format.



3. Select all five records again and display the full GenBank record (including sequences) by selecting Summary > GenBank (full) to load the data in the correct format. Once that has loaded, you can import these closely related sequences into MEGA using the Add to Alignment button at the top of the screen.

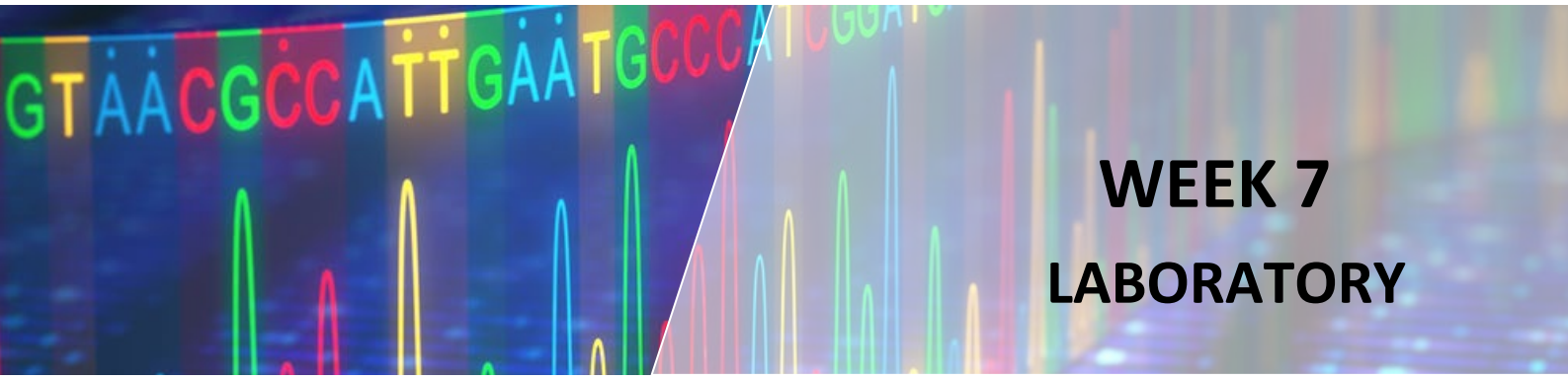


4. The related sequences will now be added to your `Alignment Explorer` window and you can repeat your alignment and phylogenetic tree construction (Exercises 2, 3, and 4) using your additional related SSU sequences. This will be particularly important for Week 11 when you have your unknown project samples and will need to identify closely related, characterised isolates. The `Alignment Explorer` window may take a few minutes to refresh.

3. Record the results of your Microbial Fuel Cell

Over the past week you have been recording the output from your Microbial Fuel Cell. Take this opportunity to take another reading and record your results in the class OneNote spreadsheet under Content Library > Soil Microbial Fuel Cell Results.

END OF WEEK 5



WEEK 7
LABORATORY

OVERVIEW

Today you will use DNA sequencing to identify your pet microbe from its 16S (SSU) rDNA sequence. The experiment is conceptually similar to your metagenomics sequencing of all bacteria in your sample with a couple of important differences. 1) You will be sequencing the 16S rRNA of a *single* species of bacteria (as you have a pure culture). 2) You will be using Sanger sequencing instead of Illumina sequencing. This type of sequencing chemistry yields much longer read lengths (>1000 base pairs compared with 50-150 base pairs from Illumina sequencing).

By sequencing the 16S (SSU) rDNA from your pet microbe, you will be able to compare its sequence to the extensive database of 16S sequence available at NCBI and determine how closely related your isolate is to a known bacterial species. These molecular data should complement the phenotypic and biochemical characterisation that you have already done. You may even be able to determine what proportion of the total microbial diversity your pet microbe constitutes in your original sample by comparing your pure culture Sanger sequencing results and metagenomic Illumina data.

To sequence the 16S (SSU) rRNA gene from your pet microbe, you will need to extract genomic DNA and then amplify the 16S rRNA gene using PCR. The amplified gene can then be sequenced using Sanger sequencing.

In brief, the steps typically used to sequence the SSU rRNA gene are:

I. Extraction of genomic DNA from a pure culture of the microbe.
II. PCR amplification of the SSU rRNA gene using universal primers.
III. Analysis of the PCR product by agarose gel electrophoresis.
IV. Purification of the amplified PCR product.
V. Sanger sequencing of the PCR product
VI. Searching sequence databases to find similar sequences.

We'll do the first two steps today. The next three steps will be done for you. You will be doing the final step by yourself in your week 11 online tutorial time.

LEARNING OBJECTIVES

- Understand the difference between Sanger sequencing of 16S rRNA sequences from pure cultures and metagenomics profiling of 16S rRNA sequences in complex environmental samples.
- Understand how DNA is isolated and sequenced from bacterial cultures.
- Apply skills developed to Week 5 to identify your pet microbe

ACTIVITIES

1. DNA extraction, biochemical characterisation, and antibiotic resistance analysis from project microbes.

2. Assessment of DNA extract quantity and quality.
3. PCR amplification of the SSU rRNA gene of project microbes.
4. Record the output of your Microbial Fuel Cell

Contents:

WEEK 7 RISK ASSESSMENT 108
WEEK 7 LABORATORY CLASS 109
1. DNA extraction from sub-cultured project isolates 109
Materials 109
Step One - Sample collection and preparation 109
Step Two - Lysis 109
Step Three - DNA binding and column washing 109
Step Four - Elution 110
2. Assessment of DNA extract quantity and quality 110
Materials 111
Procedure 111
Results 111
3. PCR amplification and sequencing of SSU rRNA genes 112
Materials 113
Procedure 113
4. Record the results of your Microbial Fuel Cell 103

WEEK 7 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk9

Hazards	Risks	Controls
General	See below	**<u>At all times</u>** follow demonstrators instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (Microorganisms, Risk Group I and Risk Group II) Bunsen burner	Infection burns	PPE (lab coats, closed in shoes & gloves as required). Adhere to aseptic techniques. Work in PC2 laboratory or BSC if required. Never leave a lit Bunsen Burner unattended or reach over an exposed flame. Keep long hair tied back.
Chemical (DNA extraction)	Irritant for skin and eyes skin or eyes, toxic if ingested.	PPE (lab coats, closed in shoes, safety glasses & gloves. SDS.
Electrical Equipment (nanodrop, PCR machine)	Electric shock/ electrocution	Avoid water/spillages when working with electrical items.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 7 LABORATORY CLASS

1. DNA extraction, biochemical characterisation, and antibiotic resistance analysis from sub-cultured project isolates

Working in your mini-group follow the protocol below to extract DNA from the microbes isolated by your group. We're using the same DNA extraction kit as we did previously so refer back to Week 2 to get an overview of the protocol. Detailed instructions are given below.

Materials:

- PureLink DNA Extraction kit
- Pipettes, tips and microfuge tubes

Step One - Sample collection and preparation

1. Flood your pure culture plate with 1 mL of phosphate buffered saline. Wash the cells off the surface of the plate with a sterile spreader. Using a sterile pipette collect your bacterial cells in a microfuge tube.
2. Centrifuge at 14,000 x*g* for 10 minutes to pellet the microbial biomass.
3. Using a pipette, remove and discard the supernatant. Avoid disturbing the pellet.
4. Resuspend the pellet in 800 µL of Buffer S1 – lysis buffer by repeated pipetting and transfer to a bead tube (with red screw cap).

Step Two - Lysis

5. Add 100 µL of Buffer S2 – lysis enhancer to the bead tube. Cap tightly and mix tube contents briefly using the vortex mixer.
6. Incubate the tube in a heat block at 65°C for ten minutes.
7. Homogenise the sample by shaking on a bead beater for 3 x 45 second cycles. *(Note: This step will be performed by the technical staff).*

Step Three - DNA binding and column washing

8. Centrifuge the tube containing your sample at 14,000 x*g* for 2 minutes.
9. Transfer up to 500 µL of supernatant to a clean microcentrifuge tube, taking care to avoid disturbing the pellet or transferring any debris.
10. Add 900 µL of Buffer S4 – Binding buffer to the supernatant and vortex briefly.
11. Open the DNA binding column (supplied in a collection tube) and add 700 µL of your sample. Cap the column, place the column-tube assembly into a microcentrifuge and centrifuge at 14,000 x*g* for 1 minute.
12. Remove the column from its collection tube and discard the flow through. Replace the column in its tube and repeat Step 11 with the remaining amount of your sample.

13. After the second spin, transfer the column (with DNA now bound to the filter inside) to the clean collection tube provided.
14. Add 500 µL of Buffer S5 – Wash buffer to the column, cap it and centrifuge for 1 minute as above.
15. Discard the flow through, replace the column in the tube and centrifuge for an additional 1 minute (this step removes residual wash buffer from the column).

Step Four - Elution

16. Transfer the column to a clean microcentrifuge tube, labelled with your group identification and the date.
17. Add 50 µL of Buffer S6 – Elution buffer to the column. Cap the column and allow it to sit at room temperature for one minute.
18. Centrifuge the column (in its microcentrifuge tube) at 14,000 x *g* for 1 minute. The DNA will now be eluted from the column into the tube and is ready for quality analysis PCR and sequencing.

Continue - Biochemical characterisation of project isolate

You will continue with the biochemical characterisation of your project isolate using the guide provided in the Appendix.

Instruction on how to perform specific biochemical tests are provided in the OneNote Notebook.

Antibiotic resistance analysis of project isolate

To perform antibiotic resistance analysis on your project isolate, you will

i. Transfer a loopful of your project isolate from your cultured plate to a microfuge tube containing 100 µL of media broth.
ii. Mix the sample by inverting the tube 8 times to create a homogenise sample.
iii. Spread plating the Of culture onto a nutrient agar
iv. Allow the culture to dry for approximately 5 minutes
v. Dispense an antibiotic disc (provided) by the technical staff) onto the culture plate.
vi. Incubate the culture overnight.

You will measure zone of clearing surrounding the antibiotic disc following the incubation.

Hint: What are you comparing this to? Control?

2. Assessment of DNA extract quantity and quality

To assess the quantity and quality of your DNA we will examine its absorbance spectrum using the Nanodrop, a UV-Vis spectrophotometer. You can use the ability of a solution of DNA to absorb light with a wavelength of 260 nm (to determine its concentration. Other

methods for checking DNA quantity and purity that we won't pursue today include agarose gel electrophoresis combined with fluorescence imaging or quantifying directly with DNA binding fluorescent dyes using Qubit technology.

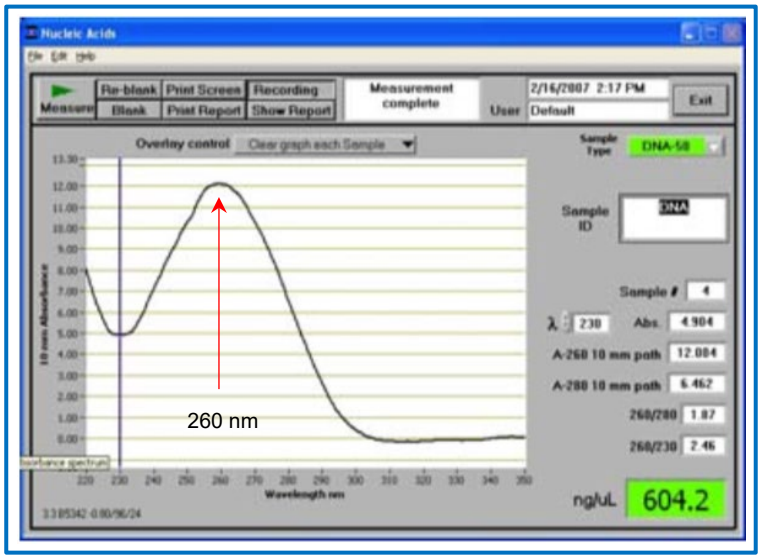
Materials:

- DNA sample from step 1 above
- Pipette and tips
- Nanodrop and Kimwipes™

Procedure:

1. To determine the absorbance of various wavelengths of electromagnetic radiation by DNA in elution buffer, first you need to see what the elution buffer looks like itself. Aliquot 2 µL of Buffer S6 – Elution buffer onto the Nanodrop stage, lower the arm and instruct the software to take a blank reading. This tells the Nanodrop that this is what the background of the sample looks like. Wipe the droplet off the stage with a Kimwipe™/tissue.

2. Now aliquot 2 µL of your DNA sample onto the Nanodrop stage and instruct the software to measure the DNA concentration. This will give you a spectrum and a concentration for your DNA.
3. Your spectrum should look something like this. The absorbance maxima (peak) of the spectrum should be at 260 nm. It is this reading that is used to calculate the DNA concentration. A 50 ng/µL solution of DNA will give you an absorbance at 260 nm of 1 so you can easily figure out DNA concentration from absorbance. The software also gives you absorbance readings at 230 nm and 280 nm. The ratio between the 260 nm and 230 nm readings (260/230 ratio) and the ratio between the 260 nm and the 280 nm readings (260/280 ratio) give you information on purity. The 260/280 ratio should be close to 1.8 for pure DNA. The 260/230 ratio should be above 1 if the sample is free from carbohydrates and phenolics. If your 260/230 and 260/280 ratios are not as expected you should not trust the concentration as measured by the Nanodrop. Note that the Nanodrop tends to slightly overestimate the DNA concentration.



Results:

Absorbance (230 nm):

Absorbance (260 nm):

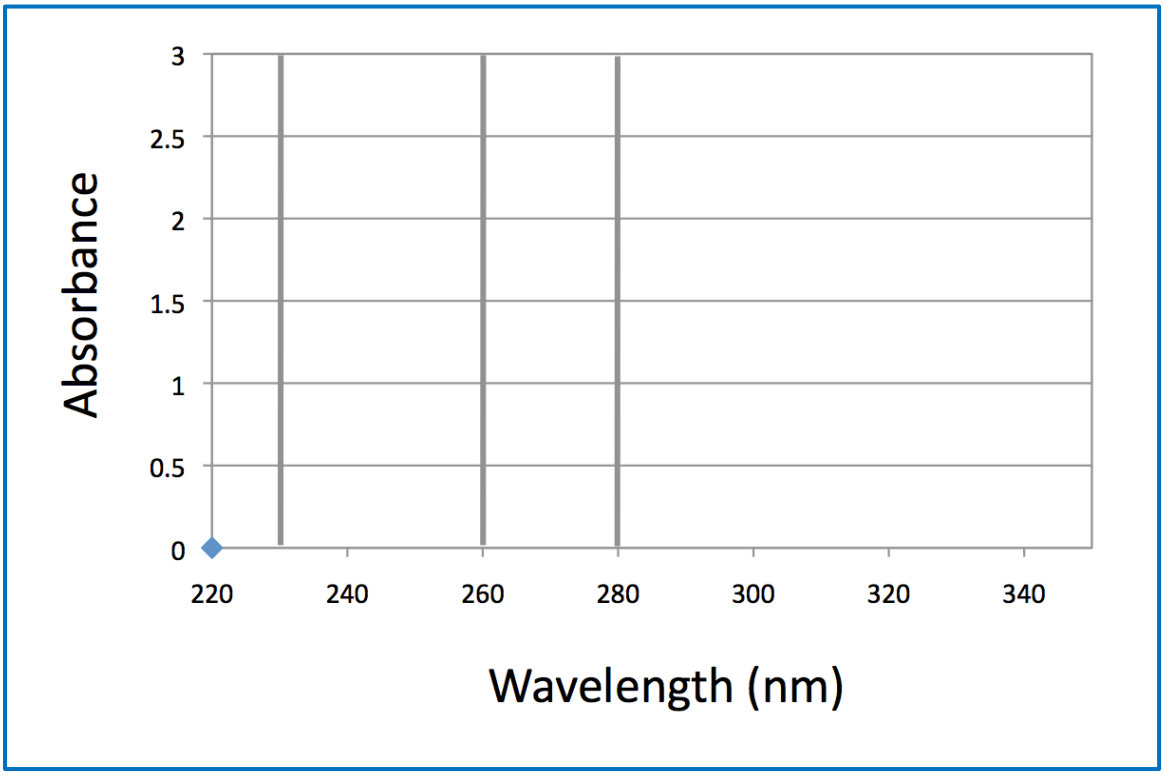
Absorbance (280 nm):

260/230 ratio:

260:280 ratio:

DNA concentration (ng/μL):

Draw the absorbance spectra of your DNA sample below:



3. PCR amplification and sequencing of SSU rRNA genes

The DNA sequence that encodes the SSU rRNA sequence is used for taxonomical classification of microbes as there are conserved regions in the sequence (due to the

conserved function of SSU rRNA) and hypervariable regions that enable us to distinguish between close relatives. The gene encoding SSU rRNA is approximately 1500 base pairs long and can be amplified in a single PCR to obtain the sequence for the whole gene. Discuss with your demonstrator what primers should be used for sequencing.
Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. The chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleosidetriphosphates (dNTPs), and modified di-deoxynucleosidetriphosphates (ddNTPs), the latter of which terminate DNA strand elongation. The pool of DNA fragments is arranged by length using electrophoresis, the ddNTPs (the end base) are fluorescently labeled for detection in automated sequencing machines (look up ABI capillary sequencing if you are interested). The data collection software converts the fluorescence signal to digital data as a chromatogram.

Materials:

- DNA sample
- 0.2 µL microfuge tubes
- PCR master mix containing
  - Forward and reverse primers (30 pmoles each)
  - dNTPs (an equal mix of dATP, dTTP, dCTP and dGTP)
  - 10 x *Taq* polymerase buffer (100 mM Tris-HCl, 500 mM KCl, 20 mM $MgCl_2$)
  - *Taq* polymerase enzyme (0.75 units)

Procedure:

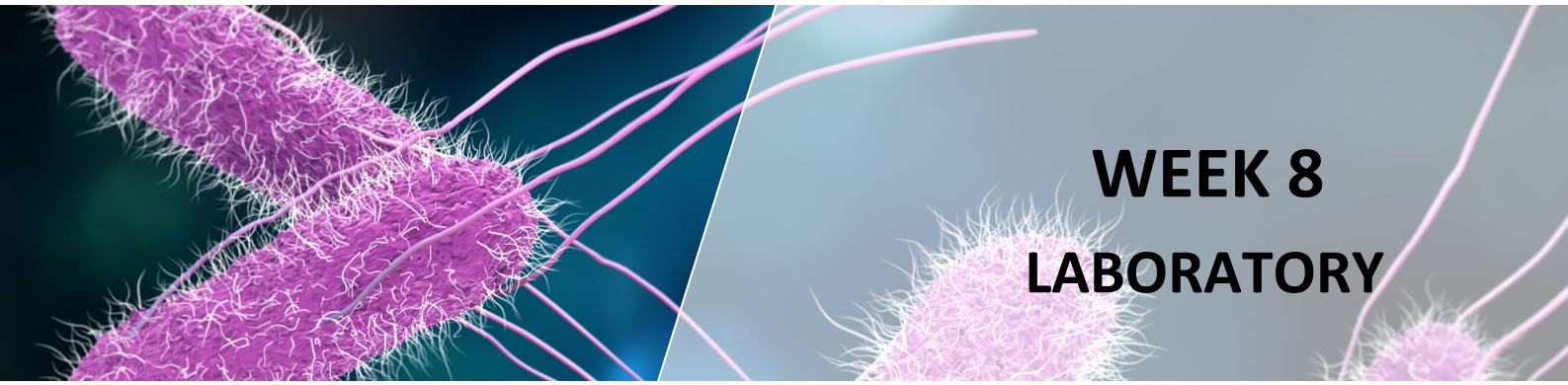
1. Label a 0.2 µL tube at the side as high as possible.
2. Add 20 µL PCR master mix.
3. Add 5 µL DNA template prepared above.
4. Place your sample on ice.
5. The samples will be collected, and the PCR will be done for you using the following PCR program: 5 min @ 94˚C, 35 cycles of 30 s @ 94˚C, 30 s @ 55˚C, 90 s @ 72˚C, followed by 10 min @ 72˚C.

One sample from each mini-group that shows a single clear PCR band will then be sent to the Ramaciotti Centre for Genomics for further analysis. The PCR product will be purified and sequenced using an appropriate primer. You will then use this sequence in the practical class next week to identify your unknown test bacterium.

4. Record the results of your Microbial Fuel Cell

Over the past two weeks you have been recording the output from your Microbial Fuel Cell. Take this opportunity to take another reading and record your results in the class OneNote spreadsheet under Content Library > Soil Microbial Fuel Cell Results. Discuss the results with your group and demonstrator.

END OF WEEK 7



WEEK 8
LABORATORY

OVERVIEW

Foods may carry a wide range of microbial contaminants, which come either from the environment in which the food was grown or processed, or from subsequent human handling. As with other habitats, the population of bacteria, which then grows and becomes dominant is selected by the particular properties (pH, composition etc.) of the food and the conditions under which it is stored. Most organisms found are harmless to the consumer's health although they may eventually cause food spoilage and economic loss.

Only a few of the possible contaminants, notably *Staphylococcus aureus, Salmonella* species, *Vibrio* species, *Clostridium botulinum*, *Clostridium perfringens*, can cause food poisoning and public health measures are directed chiefly at these organisms. The microbial population of some foods will be determined in today's exercises.

In the first exercise students at each bench will determine the predominant microbial populations in samples of two food types. The exercise will also provide information on degree of selectivity shown by the selective media used. In the second part of this practical you are provided with three 16S (SSU) rRNA sequences from bacteria isolated from the food. You will use these sequences to identify the bacteria.

LEARNING OBJECTIVES

- Apply knowledge of selective/differential media to culture microorganisms present on processed and unprocessed food.
- Understand the relative significance of isolating particular microorganisms from food.
- Understand how DNA sequencing technologies and related molecular techniques (like PCR) can be used to identify microorganisms present in food.

ACTIVITIES

1. Using basal, selective, and differential media, isolate microorganisms present in processed and unprocessed food samples.
2. Using the DNA sequences of 16S (SSU) rRNA recovered from food samples, identify the microorganism present in the food sample.

Contents:

WEEK 8 RISK ASSESSMENT 117
WEEK 8 LABORATORY CLASS 118
Materials 118
Procedure 118
Results 118
Phenotyping verses genotyping 120

WEEK 8 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk6

Hazards	Risks	Controls
General	See below	**<u>At all times</u>** follow the demonstrators' instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (Microorganisms, Risk Group I and Risk Group II) Sharps, forceps Bunsen burner	infection cuts burns	PPE (lab coats, closed in shoes & gloves as required). Adhere to aseptic techniques. Dispose of any broken/used sharp/glass items immediately in Sharps containers provided. Take care when handling forceps. Never leave a lit Bunsen Burner unattended or reach over an exposed flame. Keep long hair tied back.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

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Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 8 LABORATORY CLASS

Materials:

- 4 plates nutrient agar (per bench group)
- 4 plates mannitol salt agar (per bench group)
- 4 plates Sabouraud agar (per bench group)
- 4 plates MacConkey agar (per bench group)
- 2 sterile "Stomacher" bags (per bench group)
- 4 x 50 ml volumes physiological diluent (saline) (per group)
- 30 g of an unprocessed food in a sterile petri dish (per group)
- 30 g of a processed food in a sterile petri dish (per group)

Procedure:

Homogenise the sample of unprocessed food and processed food by placing the entire sample into a sterile "Stomacher" bag, add 50 ml diluent and homogenise for 30 seconds in the "Stomacher".

Using aseptic techniques subculture a loopful of the homogenate onto each of 2 plates of all the media supplied, then place them in a box for incubation at 30°C.

Dilute by a factor of 10, both homogenates with physiological saline, and inoculate a loopful of the diluted homogenate onto each of 2 plates of all the media supplied, then place in a box for incubation at 30°C.

Results: (Complete in next lab class)

Medium	UNPROCESSED FOOD		PROCESSED FOOD	
	Macroscopic	Microscopic	Macroscopic	Microscopic
Nutrient agar				
Mannitol salt agar				
Sabouraud agar				
MacConkey agar				

Macroscopically examine the plates set up last week. Do you have any evidence of different population sizes on the processed and unprocessed foods?

Does comparative growth on different selective media and the colony appearance of the populations suggest any qualitative differences in the populations of the two foods?

Comment on the predominant colony types on each food	

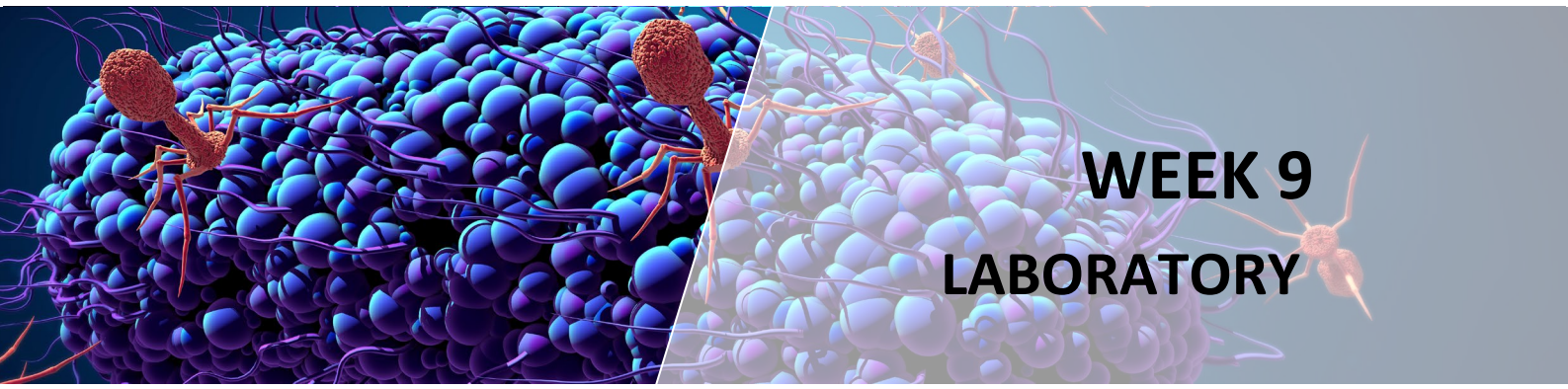
Comment on whether or not any evidence that the foods might constitute a health risk for consumers.

Phenotyping verses genotyping:
DNA was isolated from three bacteria that grew from the food sources. You are provided with three 16S sequences from these bacteria. The sequences have been uploaded in fasta format to the MICR2011 Moodle page under Week 7. Download the sequences to the lab computer and use your knowledge of 16S sequencing and analysis to identify the bacteria (hint: refer to Week 5 if you need).

Use the genotyping results to comment on whether any of the identified bacteria isolated from the foods might be capable of causing disease in humans.

Compare the genotyping results to the phenotyping results. Consider the accuracy and work involved for both techniques.

END OF WEEK 8



WEEK 9 LABORATORY

OVERVIEW

Bacteriophages are viruses that infect and multiply within bacteria. Bacteriophages play a major role in regulating the abundance of bacteria in the environment, including the human body. Bacteriophages are used to prevent the souring of petroleum resources caused by sulphate reducing bacteria and to prevent, and even treat, bacterial infection. This week you will learn to quantify bacteriophage and examine bacteriophage mediated transfer of genes between bacteria in a process called transduction.

LEARNING OBJECTIVES

- Bacteriophages are obligate intracellular parasites and do not form independent colonies. This poses challenges for standard culture-based enumeration. Student's should understand how to cultivate and enumerate bacteriophages using plaque assays.
- Horizontal gene transfer allows bacteria to exchange DNA between species and accelerates bacterial evolution. Bacteriophages facilitate horizontal gene transfer through transduction and students should understand how this occurs and how we can demonstrate transduction using genetic markers like auxotrophy.

ACTIVITIES

1. Propagation and enumeration of bacteriophage
2. Horizontal gene transfer by bacteriophage transduction

Contents:

WEEK 9 RISK ASSESSMENT 112
WEEK 9 LABORATORY CLASS 113
1. Bacteriophage propagation and enumeration 113
Materials 114
Procedure 114
Dilution of the phage stock 114
Phage absorption and overlaying agar 114
2. Gene transfer by transduction 115
Materials 116
Procedure 116

WEEK 9 RISK ASSESSMENT

BABS Teaching Laboratory Student Risk Assessment	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMF_Wk8

Hazards	Risks	Controls
General	See below	**At all times** follow demonstrators instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (Microorganisms, Risk Group I and Risk Group II) Sharps, forceps Bunsen burner	infection cuts burns	PPE (lab coats, closed in shoes & gloves as required). Adhere to aseptic techniques. Dispose of any broken/used sharp/glass items immediately in Sharps containers provided. Take care when handling forceps. Never leave a lit Bunsen Burner unattended or reach over an exposed flame. Keep long hair tied back.

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Clean up and waste disposal

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Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 9 LABORATORY CLASS

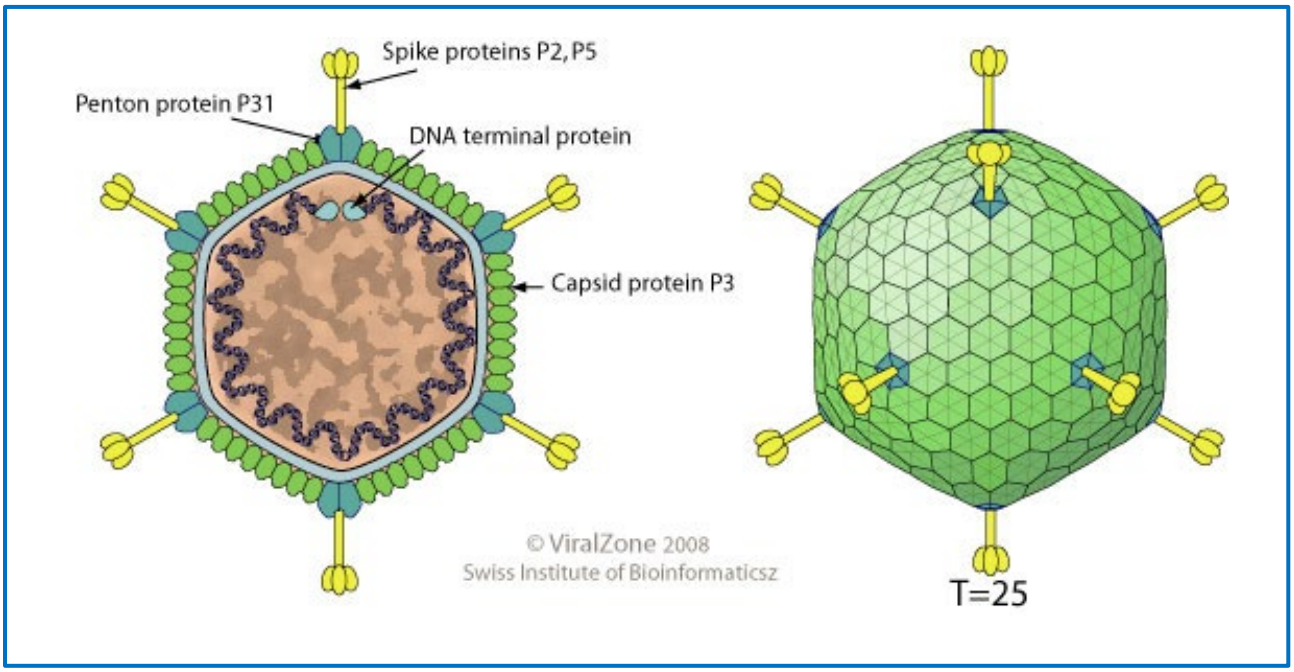
1. Bacteriophage propagation and enumeration

Bacteriophage infection consists of a number of sequential events (refer to lecture by Prof. Peter White). The phage is first absorbed by the host cell, then replicates and assembles into mature virions, encapsidating the the phage DNA or RNA. Typically, this growth period is about 30 minutes and phage particles multiply in the cell resulting in a few hundred new viruses. Infection usually ends when the bacterial cell is lysed by the phage. The viral particles released are capable of infecting new susceptible hosts and the so called “lytic cycle” starts all over again. Phages can also integrate into the bacterial host genome to form a prophage, this is known as lysogeny.

The method used to enumerate a bacteriophage suspension is very similar in principle to that used for the enumeration of a bacterial culture. In both cases 10-fold serial dilutions of the microorganism are made and then plated onto agar plates. However, bacteriophages need a bacterial strain (host) in which to replicate. Infection of the host strain results in the formation of “plaques” or clear zones in the areas where cells have been lysed by the phage. As with a bacterial count, where each colony arises from a single cell, in the plaque assay each plaque arises from a single phage particle. This is designated a plaque forming unit (PFU) and is used to quantitate the number of infective phage particles. This week we will be using this theory to enumerate a phage called PRD1 on a lawn of the bacterium *Salmonella typhimurium*.

PRD1 belongs to a family of lipid-containing double capsid bacteriophages that infect both Gram-negative and Gram-positive bacteria. It has one genus, Tectivirus and unlike many phages PRD1 has no real visible tail (see figure below). It has a virion size of about 66 nm with apical spikes of 20 nm. The capsid encloses an inner membrane vesicle within which the genomic DNA is coiled.

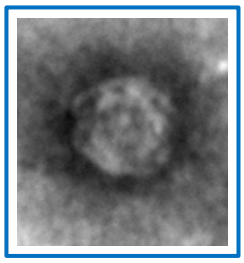
One thing to note, PRD1 plaques are larger than P1 phage plaques (used for the second experiment) – see if you can spot this in the laboratory experiment.
The genome is a linear double stranded DNA molecule of 15 kb flanked by inverted repeats. It encodes for 30 proteins and replication is protein-primed.



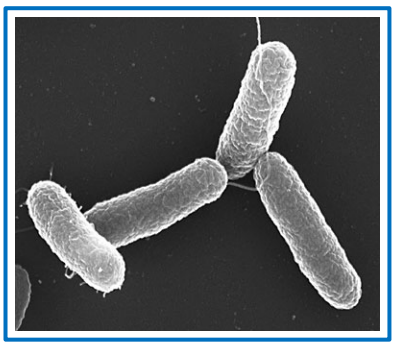
Materials:

Per mini group of students:

- 1 x 0.5 mL PRD1 bacteriophage stock



- 1 mL 24 h Z broth culture of *Salmonella typhimurium LT2*



- 10 mL Z broth
- 4 x Z agar plates (1 for control, 1 for each phage dilution to be plated)
- 4 x 5 mL 0.7% Z Soft Agar at 50°C

Procedure: Work in mini groups.

Dilution of the phage stock

Students are required to sketch the dilution procedures in their lab book before proceeding. The phage stock is to be diluted in Z broth using a series of 3 x 10-fold dilutions, starting with adding 0.1 mL of the original phage stock to a 0.9 mL Z broth (1 in 10 dilution).

Phage absorption and overlaying agar

1. Label all tubes and plates.
2. Obtain one Z soft agar from water bath, and immediately add 0.1 mL of *S. typhimurium* LT2 broth culture, and 0.1 mL of 10^{-1} PRD1 phage dilution. Do not take the soft agar out of water bath unless you are using them immediately or it will set. Do not leave soft agar standing in the 50°C water bath following addition of the bacterial cells and/or phage because they will die.
3. Briefly vortex the soft agar 5 times to mix the bacterial cells and phage particles, and pour onto the centre of the Z agar plate. With the plate sitting flat on the bench, GENTLY move it from side to side to further mix the cells and phage particles until the soft agar is evenly spread on the surface of the plate. Do not swirl the plate.
4. Allow the soft agar layer to set for at least 10 min.

5. Repeat steps 2 to 5 with the 10^{-2} and 10^{-3} phage dilutions.
6. The remaining Z soft agar and Z agar plates will be used as a control. To the soft agar, add 0.1 mL of *S. typhimurium* LT2 culture only (i.e. no phage). Mix as described in step 3 and pour the soft agar onto a Z agar plate and allow the soft agar to set for at least 10 min.
7. Hand the plates to your demonstrator who will incubate them at 37°C for 24 hours before storing them at 4˚C. Results will be analysed in next week's practical class.

2. Gene transfer by transduction

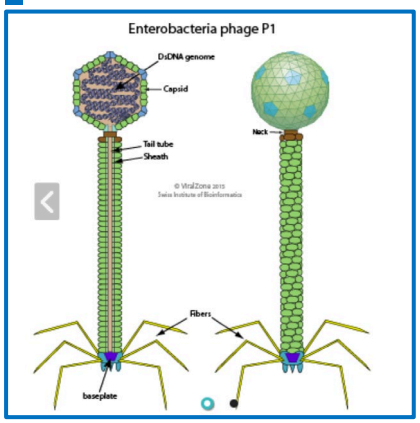
Apart from infecting and killing bacteria, bacteriophages also play an important role in gene transfer. Some bacteriophages are able to transfer bacterial DNA from one bacterial strain to another. In this practical, we will transfer genes from a donor bacterium to a recipient bacterium, mediated through the P1 bacteriophage P1kc, a generalised transducing phage. As a recipient we will use a strain of E. coli that is unable to live without organic supplements (an auxotroph, E. coli strain AB2828). This strain requires phenylalanine, tyrosine, tryptophan, p-aminobenzoate and p-hydroxybenzoate for growth. The auxotrophy is due to a mutation in the gene aroE blocking the aromatic amino acid biosynthesis pathway. For bacteriophage P1kc to be used as a vector in DNA transfer from a donor to a recipient strain, the phage must infect and be propagated in the donor E. coil strain (AN180) to pick up donor DNA, in this case an aroE+ E. coli strain. Transfer of the DNA then takes place through a second phage infection of the recipient strain.

Bacteriophage P1 (see figure below) was isolated in 1951 by Giuseppe Bertani. P1 has been widely used to construct new bacterial strains and was used extensively to map the E. coli genome. P1 has served as a template virus for different aspects of biology research such as DNA restriction modification, site-specific recombination, plasmid replication, partition systems and plasmid incompatibility. Phage P1 is a virulent bacteriophage of the P1-like virus genus, in the family Myoviridae. This phage can quickly turn an E. coli cell into a P1-producing factory that releases hundreds of phages when the cell ruptures.

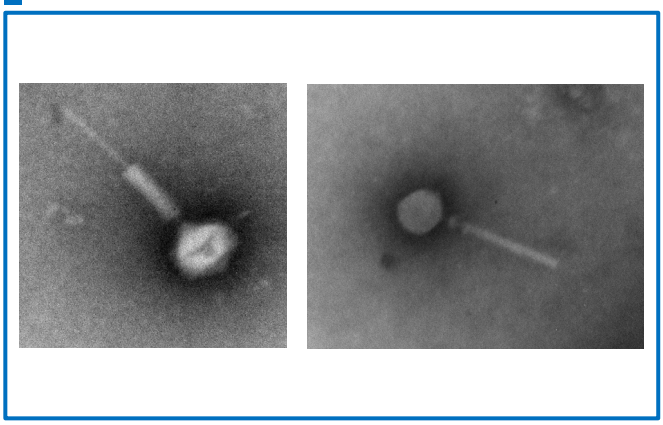
[#]Bertani, G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. 62:293-300.

The P1 phage has a 94 kb genome that encodes at least 117 genes which are organised in 45 operons. In the capsid, DNA is double stranded, linear and terminally redundant. The terminal region is where foreign pieces of DNA can be inserted as the genes in this region are non-essential. Once the phage has entered into the host the genome circularises and replicates as a plasmid, but only during the lysogenic cycle.

A



B



Panel A shows a schematic of the structure of the bacteriophage P1, with head and tail structures. The icosahedral capsid can house the 94 kb genome. Phage P1 exhibits the classical morphology of a bacterial virus. It has an icosahedral head and a 220 nm long inflexible tail, with a tube surrounded by a contractile sheath. The virus possesses a baseplate at the bottom of its structure where six bent tail fibres are located. Panel B shows two images of the same P1 phage used in this practical (P1*kc*) taken at the UNSW Electron Microscopy Unit.

Materials:

Per mini group of students:

- 1 x 1 mL P1Kc phage stock prepared from *E. coli* AN180 (*aroE*$^+$)
- 1 x 1 mL Z broth of *E. coli* strain AB2828 Δ*aroE* at 10^9 cells/mL
- 1 x 10 mL Z broth
- 1 x 5 mL Z broth
- 2 x Medium E selection plates (1 for each cell suspension)
- 1 x 9 mL Medium A for washing

Procedure: Work in mini-groups.

You have been provided with a lysate of the P1 phage P1*kc*, and a culture of the recipient *E. coli* strain AB2828 Δ*aroE*, that is missing the *aroE* gene.

1. To 4 mL of Z broth in a 12 mL sterile centrifuge tube, add 1 mL P1*kc* (AN180) lysate and 0.1 mL *E. coli* strain AB2828. Label as “Transduction”.
2. To 5 mL Z broth in a 12 mL sterile centrifuge tube, add 0.1 mL *E. coli* strain AB2828 Δ*aroE* only. Label as “control”.
3. Incubate at 37°C in a water bath for 60 min (no shaking needed).
4. Centrifuge the tubes at 14,000 *xg* for 15 min, and re-suspend the cell pellet in 2 mL of Medium A. Be care to remove as much free bacteriophage (the supernatant) as possible.
5. Repeat step 4 to remove as much free bacteriophage as possible.
6. Spread 0.1 mL cell suspension on Medium E selection plate: one for the control and one for the transduction. Label accordingly.
7. Hand the plates to your demonstrator who will incubate plates for 48 h at 37°C and then store them at 4˚C. Results will be analysed in next week’s practical class.

What type of phage is P1*kc*?

How is a bacteriophage plaque formed on an agar plate?

In centrifugation, what is the difference between rcf and rpm?

END OF WEEK 9

WEEK 10
LABORATORY

OVERVIEW

Today you will be looking at how we can track the spread of viruses (or many infectious agents) during an outbreak. Specifically, you'll be looking at a case study of Ebola infection and using ELISA analysis of blood serum samples to determine the source of the infection.

LEARNING OBJECTIVES

- Understand how viral pathogens can be detected and monitored in infected individuals using an ELISA.
- Understand how the combination of molecular detection techniques and patient meta-data is used determine the epidemiology of an infectious agent and trace the source of infection in an outbreak.

ACTIVITIES

1. Background reading on Ebola infection and disease progression
2. Background to the epidemiological investigation
3. ELISA analysis of mock patient serum
4. Tasks for the investigating team – determine the source of the outbreak
5. Record the results from you phage enumeration experiment

Contents:

WEEK 10 RISK ASSSSMENT 120
WEEK 10 LABORATORY CLASS 121
1. Background 121
Ebola virus 122
History 122
Transmission 123
Signs and Symptoms 124
Control 124
2. Epidemiological investigation 125
3. ELISA analysis of mock serum samples 127
Materials 128
Procedure 128
4. Tasks for the investigation team 129
Tasks 129
Questions 129
5. Record results for PRD-1 phage enumeration and P1*kc* transduction experiments 125

WEEK 10 RISK ASSSSMENT

BABS Teaching Laboratory	UNSW THE UNIVERSITY OF NEW SOUTH WALES	MICR2011_RMR_Wk12
Student Risk Assessment		

Hazards	Risks	Controls
General	See below	**At all times** follow demonstrators instructions when handling all biological substances, chemicals and equipment items. Notify demonstrator immediately of any spills or other incidents. Proper handwashing with antibacterial handwash before leaving the laboratory.
Biological agent (human samples eg urine, blood, tissue)	Infection Spills	PPE (lab coats, closed in shoes & gloves as required) and Safety glasses when handling human specimens. Adhere to aseptic techniques. Work in PC2 laboratory or BSC if required. Dispose of any broken/used sharp/glass items immediately in the Sharps containers provided.
Chemical (TMB solution)	Non-hazardous	PPE (lab coats, closed in shoes, safety glasses & gloves. SDS.

Personal Protective Equipment

Emergency Procedures

In the event of an alarm sounding, stop the experiment, turn of electrical equipment and wait for confirmation from demonstrators to evacuate. Then pack up your bags and wash your hands. Follow the instructions of the demonstrators regarding exits and assembly points. The BioScience assembly point is in the lawn in front of the Chancellery. In the event of an injury inform the demonstrator. First aiders and contact details are on display by the lifts.

Clean up and waste disposal

All used microfuge tubes should be placed in the biohazard bins. Used test tubes should be placed in the appropriate container on the front bench of the lab. All solutions should be placed in the appropriate liquid waste containers. Used pipette tips should be placed in approved biohazard sharps containers.

Declaration – will be checked by demonstrator at the beginning of the practical class

I have read and understand the safety requirements for this practical class and I will observe these requirements.

Signature:……Date:……
Student number:……..

WEEK 10 LABORATORY CLASS

1. Background

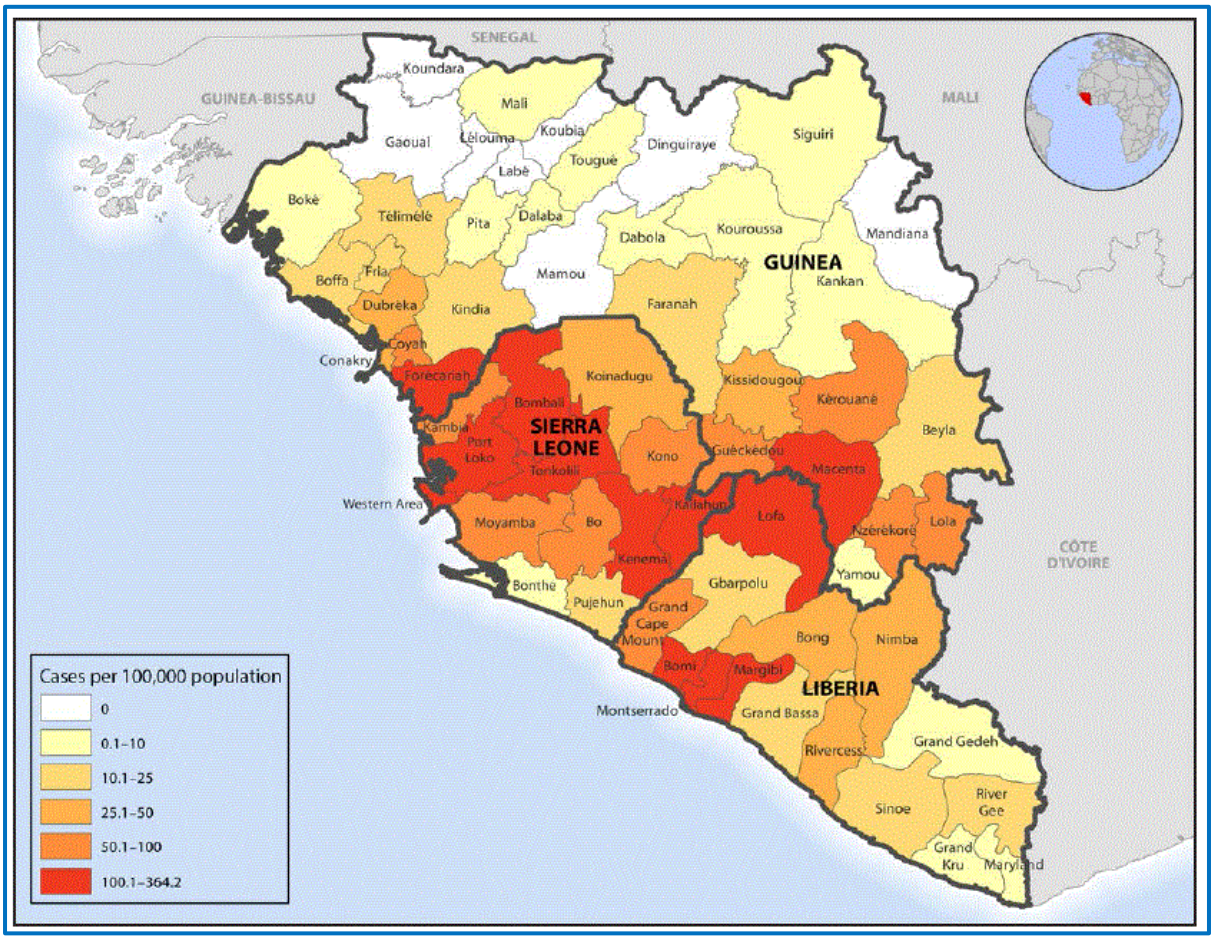
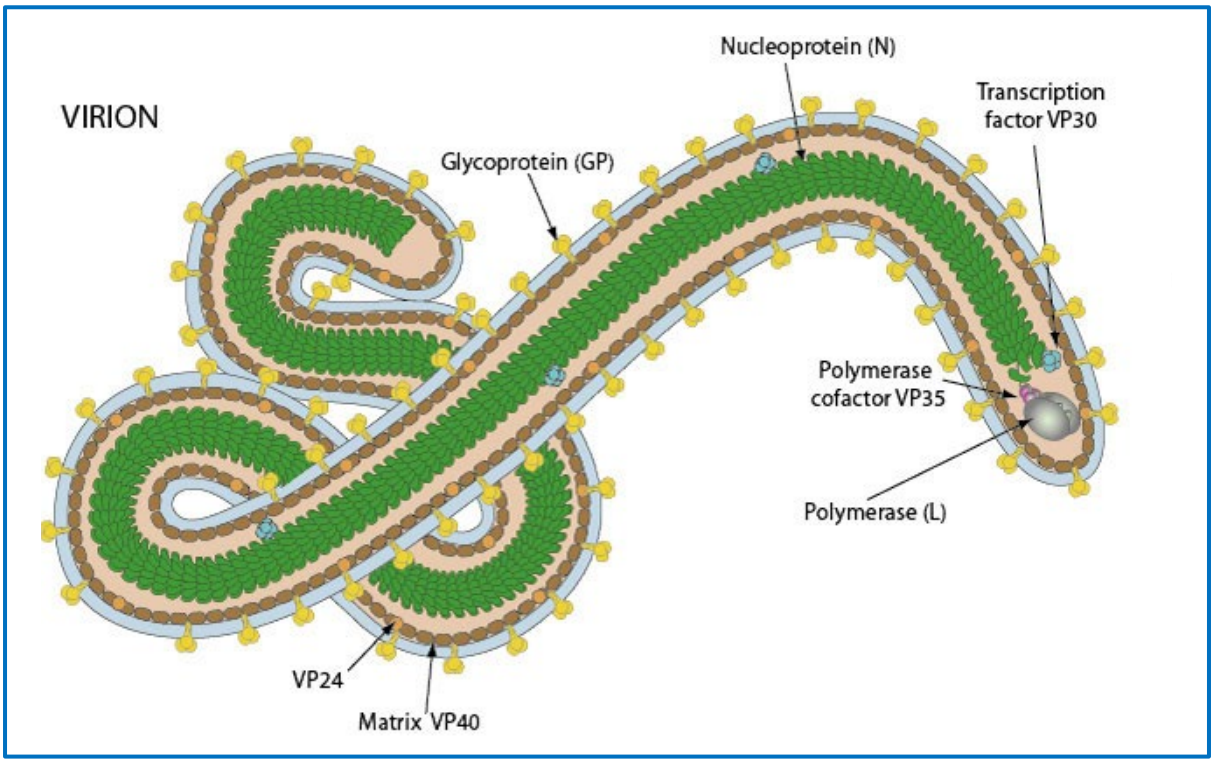


Figure 1. Reported active human transmission in 2014-2016 (CDC website)
Background by Professor Vincent Racaniello, author of “This week in Virology”
Reference: http://www.microbe.tv/twiv/twiv-341/
To see more about Vincent: http://www.virology.ws/about/

Ebola virus:



The widespread outbreaks of Ebola virus in the West African nations, together with the fatality rate of up to 90% have propelled this virus into the limelight in recent years. What is this virus and where did it come from?

History:

Ebola virus was first identified in 1976 and is named after a river in the Democratic Republic of the Congo (formerly Zaire). Outbreaks have been sporadic since its discovery, and several strains have been identified in outbreaks across the African continent. Between 2014 and 2016 there was the largest Ebola outbreak in history, resulting in the most human fatalities since the discovery of the virus. Following the first confirmed case in March 2014, 28,637 cases were reported in the outbreak that spanned the West African nations of Sierra Leone, Guinea, Nigeria, Mali and Liberia. During the outbreak 11,315 people died, including one man that died in the US after travelling from Liberia for treatment.

Ebola resides in rainforests in Africa, and three fruit bats have been identified as the animal reservoirs that carry the virus asymptomatically. Ebola virus can infect non-human primates including apes, gorillas and chimpanzees, and other wild animals including pigs and deer.

To date five Ebola virus species have been identified, with four known to cause human disease. The human pathogenic strains include Zaire ebolavirus, Sudan ebolavirus, Taï Forest ebolavirus, (formerly Côte d'Ivoire ebolavirus), and Bundibugyo ebolavirus. The fifth species, Reston ebolavirus, causes disease in non-human primates and farmed animals including pigs, but appears to be non-pathogenic in humans. In contrast, the Zaire ebolavirus is highly pathogenic in humans with fatality rates of up to 90%.

Outside of Africa there have been several cases of Ebola virus reported in countries including the United States, Russia, Italy and Spain. There have been reports of isolated laboratory infections through occupational exposure in the United Kingdom and Russia. In the United States, Reston ebolavirus has been detected in imported monkeys, with some transmission to humans, resulting in asymptomatic infections. One Spanish nurse was diagnosed with Ebola virus after caring for a priest repatriated from West Africa, although it was reported that she may have cleared the virus spontaneously.

Ebola is a member of the *Filoviridae* family, from the genus *Ebolavirus*. The negative sense single-stranded RNA genome is around 19 kb in size and encodes seven proteins. The genome is enclosed in a filamentous capsid of helical structure that is around 900 nm long and 80 nm wide. The 'L gene' encodes the 'Large' protein, which includes the viral polymerase domain and other enzymatic domains needed for replication. The viral envelope glycoprotein is involved in host cell binding and entry.

Transmission:

Ebola virus is transmitted through direct contact with bodily fluids and aerosols from infected individuals and the use of unsterilised needles. As Ebola causes haemorrhagic disease, the families of infected individuals often become infected through unprotected contact with blood, vomit, urine and faeces when caring for patients.

There are several reports of people becoming infected while attending traditional funerals of Ebola victims, and in health-care workers treating infected patients. Sexual transmission has been reported, and Ebola virus has been detected in semen. Transmission from mother to baby through breast milk has also been reported.

Zoonotic transmission (from animals to humans) can occur from contact with infected fruit bats, primates and swine. The practice of hunting and eating bushmeat (meat from wild animals including bats and gorillas) is known to be a high-risk factor in zoonotic transmission of Ebola virus. In 2015, transmission to humans following consumption of infected dog meat in Nigeria was reported.

Signs and Symptoms:

Symptoms of pathogenic Ebola infection include fever, severe headaches, muscle pain, weakness, fatigue, diarrhoea, vomiting and severe haemorrhaging. The incubation period is usually between 2 and 21 days after exposure to the virus, with an average of 8-10 days before the onset of symptoms.

The first symptoms usually appear around 8-10 days after exposure and include headaches, fever, fatigue and muscle soreness. Around day 10 the patient develops a sudden high fever and starts to vomit blood. By day 11 bruising, brain damage and bleeding from the nose, mouth, eyes and anus occur. The final stages are around day 12 and include loss of consciousness, seizures, shock, respiratory failure, massive internal bleeding, and finally death.

Control:

Currently there are no FDA approved vaccines or antiviral drugs available for the treatment of Ebola virus infections. There are several experimental vaccines under development, however these have not yet been fully tested for safety and efficacy. Vaccine candidates in clinical trials include the Zaire ebolavirus (ZEBOV) glycoprotein, which is vectored in the recombinant vesicular stomatitis virus (rVSV). The rVSV-ZEBOV vaccine was shown to be 70-100% effective at preventing disease in the latest clinical trial in December 2016.

People at risk of Ebola virus exposure should wear full protective clothing including masks, gowns, goggles, gloves and closed footwear with no skin left exposed. Protective wear must also be used when handling exposed objects that may have been contaminated with the virus. It is recommended that medical personnel undergo a 12-day biosafety training course before treating patients. Disinfection of contaminated equipment can be carried out using heat (boiling for five minutes or 60°C for one hour), by using detergents, alcohol-based products or bleach. Bushmeat is an important food source for some African people, and should be hunted and handled with full protective clothing and cooked thoroughly before consumption. Direct contact with infected corpses at traditional West African funerals should be avoided and quarantine is required for all individuals infected and possibly exposed to the virus.

While there is no specific treatment available for Ebola virus patients, supportive therapy includes fluid replacement, analgesics and replacement of coagulation factors to prevent fatal hemorrhaging.

2. Epidemiological investigation

September 21st 2018

As part of an Australian foreign aid venture, the Federal Government committed $4.5 million to a two-year project aimed at improving Ebola virus diagnosis in Sierra Leone. The key West African company involved in the project was WABIRT (West African Biotechnology and Industrial Research Technology), a local company that produced a number of virology molecular and serological testing kits, as well as a number of other biotech portfolios. In addition, five major hospitals in and around Freetown, Sierra Leone were recipients of the Australian Foreign Aid funded diagnostic tests and testing equipment, developed at UNSW as a sensitive Ebola antigen detection kit, providing rapid and early detection over antibody tests. These were provided free as part of the aid funds.

As part of the project, a foreign aid team including Prof Peter White, Mr. John Wilson and five other AusAID delegates left Australia and travelled to Freetown, Sierra Leone via Nairobi, Kenya on Kenyan Airways. The AusAID team transfer from flight KQ887 to flight KQ504 in Nairobi where they are joined by epidemiologists Dr Jai Tree, Mr Brandon Sy, and Dr Tendai Idowu working with UNSW and the Medical Research Council (MRC), UK. They have been collecting samples from an outbreak of Rift Valley Fever in neighboring western Uganda, and have travelled from Bundibugyo, Uganda to Nairobi Airport. The teams travel on flight KQ504 to Freetown, Sierra Leone. The aim of the trip was to determine how well the aid project is going and how well the new kits were performing. The epidemiological team also aim to collect serum samples from the Ebola outbreak in Sierra Leone and deliver them to MRC Unit in The Gambia where they will be sequenced.

The AusAID team want to determine if the Australian diagnostic tests were more capable of detecting early infection of Ebola virus compared to other available kits/tests. To achieve this aim, the team are to liaise with the local participants (hospitals and WABIRT), and also to determine if the equipment supplied to the hospitals through the foreign aid program was being well used.

September 23rd 2018

The team hears reports of potential Ebola cases in Kenema and Kambia, so decide to split into 3 groups to distribute the diagnostic tests and provide support for testing.

Group 1 travel to Kenema with WABIRT technical staff. The group includes:

- Dr Rowena Benton
- Dr Jenny Morgan
- Mr. John Wilson

Group 2 travel to Kambia with researchers from University of Makeni and Njala University. The groups includes:

- Prof Bill Roberts
- Dr Afia Ayodele,
- Dr Gillian Brown
- Subira Arendse

Group 3 remains in Freetown where they work with staff and officials at Abanita Hospital. The group includes:

- Prof Peter White
- Prof Zuri Babatunde
- Prof Leighton Scott
- Dr Jai Tree
- Mr Brandon Sy
- Dr Tendai Idowu

September 24th 2018

After collecting samples from the Abanita Hosptial in Freetown, the epidemiological research team travel to the MRC Unit in The Gambia to deliver samples of Rift Valley Virus and Ebola for sequencing. The experiments will take 3 weeks, so Brandon Sy and Dr Tendai Idowu leave Freetown early for the MRC Unit and Dr Jai Tree stays behind in Freetown to attend the Grand Opening of the WABIRT laboratories at the end of the week.

September 28th 2018

After observing and supporting the implementation of the new testing kits in Kenema and Kambia, the AusAID teams return to Freetown for the Grand Opening of the new Australian Foreign Aid funded WABIRT laboratories. Over 120 invited guests attend including those from WABIRT, University of Makeni, Njala University, the Sierra Leone Ministry of Health, the Australian embassy, the Sierra Leone government, a number of local hospitals and many other sources.

Both Dr Rowena Benton and Dr Jenny Morgan have had headaches and sore muscles for the previous 2 days. They do not attend the Opening and Dr Jenny Morgan is admitted to Abanita Hospital in the early afternoon with some blood in her vomit.

On examination she was unwell, with a high fever of 39.9°C and signs of bruising and blood from her eyes and nose. Antigen testing (using the new Australian antigen assay) of a blood sample reveals that Jenny had been infected with Ebola virus. Over the subsequent 12 hours Jenny's haemorrhagic symptoms worsens accompanied by seizures and loss of consciousness. From there, her condition deteriorates and she died at 11:45 pm the following day.

October 6th 2018

Seven people who attended The Grand Opening became ill with Ebola-like symptoms and are admitted to Abanita Hosptial. These include:

Prof Peter White
Dr Afia Ayodele
Prof Zuri Babatunde
Prof Subira Arendse
Dr Masamba Ejiro
Mr Amadi Afolabi
Dr Tinashe Kayode

Due to the death of Dr Jenny Morgan, your team has been assigned by the Australian Embassy to investigate the outbreak. A West African official (your demonstrator) has been assigned to help the investigation by answering your questions where possible and

obtaining samples under your direction. The official may also help you to trace the whereabouts of specific individuals prior to the Opening. To aid your investigation you have been given a state-of-the-art Enzyme Linked ImmunoSorbent Assay or ELISA (developed at UNSW and commercialised through UNSW Innovations, the commercial arm of the University), which can detect with high sensitivity Ebola antigens (the Ebola virion) in blood samples.

Aim: To trace outbreak of Ebola virus, and identify the source of the outbreak

- First of all find how many of the seven attendees of the Grand Opening who became ill have the Ebola virus. You will also test sera from Drs Benton and Morgan. This will help you become familiar with the ELISA. Include a positive and negative control.

Suspected Cases	Ebola Test Result
1 Dr Rowena Benton	
2 Dr Jenny Morgan	
3 Prof Peter White	
4 Dr Afia Ayodele	
5 Prof Zuri Babatunde	
6 Prof Subira Arendse	
7 Dr Masamba Ejiro	
8 Mr Amadi Afolabi	
9 Dr Tinashe Kayode	
Positive control	
Negative control	

3. ELISA analysis of mock serum samples

This lab includes a mock ELISA test for detecting the presence of Ebola virus antigen from blood (serum) samples. Each team has a number of serum samples available for testing. The ELISA will be used to test for Ebola virus.

Working in your mini groups, the samples will be divided between the four mini groups as below:

Mini group 1: 2 samples from people displaying symptoms after the Grand Opening (S1 – S2) plus 15 samples from flight KQ504.

Mini group 2: 2 samples from people displaying symptoms after the Grand Opening (S3 – S4) plus 15 samples from Abanita hospital.

Mini group 3: 2 samples from people displaying symptoms after the Grand Opening (S5 – S6) plus 10 samples from the Kambia Team.

Mini group 4: 3 samples from people displaying symptoms after the Grand Opening (S7 – S9) plus 6 samples from the Kenema Team.

Materials:

- Pipettes and tips
- Plates
- A positive test sample and a negative test sample
- Paper towels
- Antibody solution
- Washing solution
- Colour reagent solution (TMB)

On the 6th October blood samples are taken from those people in Freetown that came into contact with Drs Morgan and Benton, and attendees from The Opening. You have blodd samples from:

- Dr Jenny Morgan and Dr Rowena Benton
- 6 samples from the Kenema Team
- 10 samples from the Kambia Team
- 15 samples from the Abanita Hospital
- 7 people displaying symptoms after the Grand Opening
- 15 samples from passengers on flight KQ504 that were still in Freetown

- 120 samples from The Opening are not available for testing.
- 125 samples from flight KQ504 are also not available.

Procedure:

1. Label the tubes appropriately using the marker provided.
2. Your team will run an ELISA assay on your samples using the microtiter plates provided.

   Initially each team will do one positive and one negative control. Add 100 µl of a positive test solution into two wells and 100 µl of a negative test solution into a different set of two wells. Be sure to write down which wells are positive and which are negative controls. 100µl should fill one third of the well.
3. Add 100 µl of your nine suspected Ebola virus samples into each of two wells. Record which wells contain your samples.
4. After adding the samples leave the plate on the lab table undisturbed for 5 minutes.
5. Shake off the fluid into a nearby sink or designated container, making sure that the fluid has emptied from each well. Tap the plate upside down onto the paper towel to remove any excess liquid or bubbles.
6. Wash the plate using 150µl of washing solution in each well. Repeat a total of three times.
7. Remove excess solution and bubbles by again tapping the plate face down onto a paper towel.
8. Add 100 µl of the enzyme conjugated antibody solution to each well.
9. Allow for 5 minutes incubation time on the lab table and then shake off the fluid.
10. Repeat steps 6 and 7.
11. Add 100 µl of the colourising (TMB peroxidase substrate) solution to each well.

12. Record your observations after 5 minutes.
13. Each group will record the results of the tests and make a list of the infected
14. samples. Use the results of the tests to aid in your investigation.

4. Tasks for the investigation team

Once you are familiar with the ELISA and have taken careful note of the positive results, choose samples from the outbreak for further testing. State your reasoning below for choosing the samples. You should have around 30 tests left. You may need the help of other groups to finally determine the source of the outbreak.

At the end of the investigation your findings will be pooled with other teams involved in the outbreak to give a fuller picture of the epidemiological spread of the virus.

Tasks:

- Sample selection – what samples will you test and why? Are there any samples missing that could give you more information?
- Where possible try to determine who the Ebola virus positive people were infected by
- Try to trace the epidemiological route of the outbreak (a flow diagram of events may help).
- Trace the person who was the source of the outbreak
- Implement recommendations based on your findings to help slow the spread of Ebola virus

Questions:

- In Dr Jenny's Morgan's case, why is her death not surprising?
- What are the advantages of the ability of detecting viral antigen, rather than antibodies directed against the Ebola virus?
- What is the current status of the Ebola vaccine? (you can use the internet to find the answer)

5. Record results for PRD-1 phage enumeration and P1*kc* transduction experiments

Using results from your mini-group and groups within your demonstrator group, record the plaque forming units (PFU) for each dilution of the PRD-1 phage stock below.

Phage dilution ($1x10^x$)	10	10	10	10	10	10	10	10
PFU (per 0.1 mL)								

What is the concentration of the PRD-1 phage stock solution (expressed as PFU/ml)?

Check for colonies from your P1*kc* transduction experiment last week and answer the questions below.

Did you observe any colonies on your “control” or “transduction” plates?

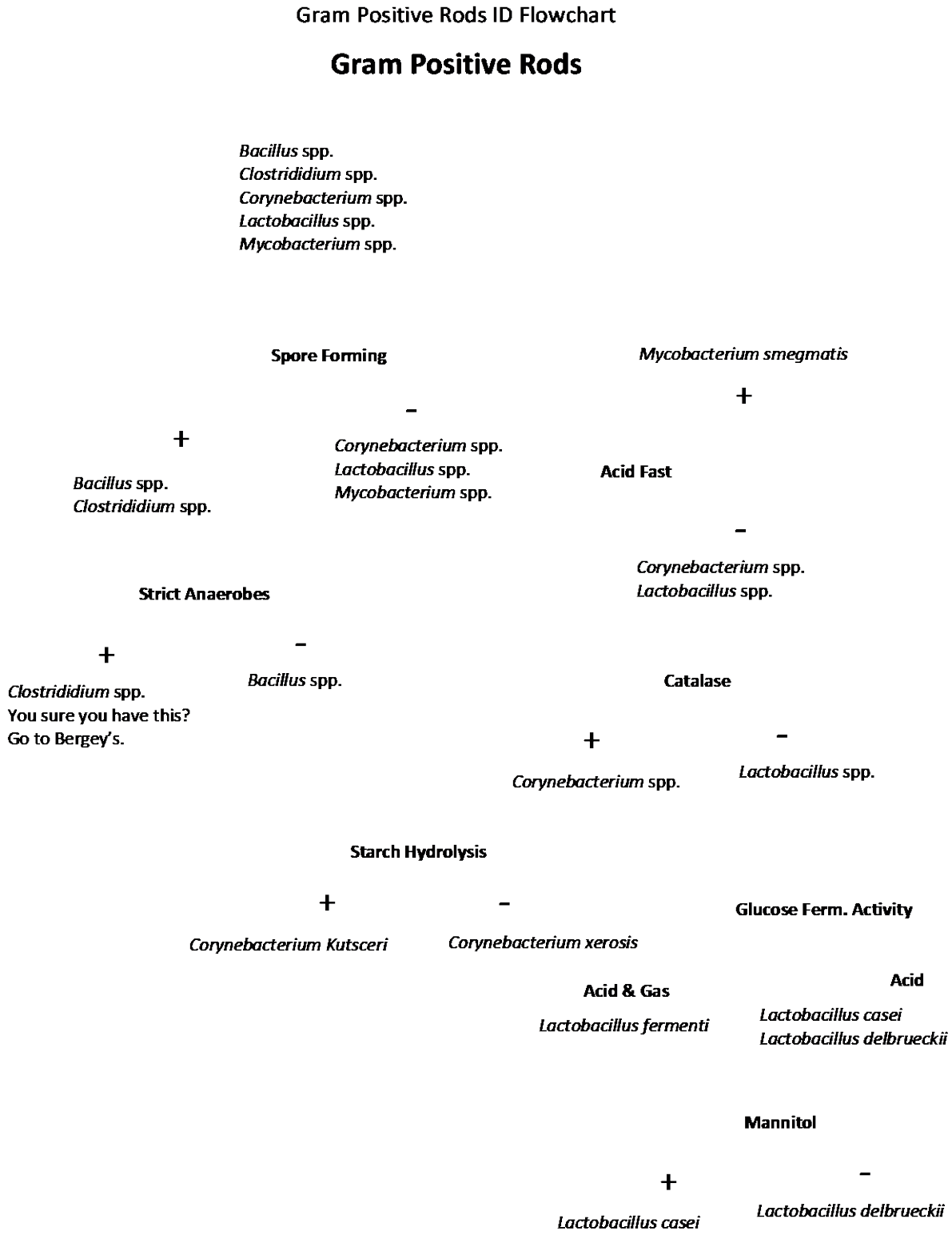
What is the selection provided by the Minimal E plates that prevents growth of the AB12828 Δ*aroE* strain?

What is the presumed genotype of any colonies growing on your “transduction” plates?

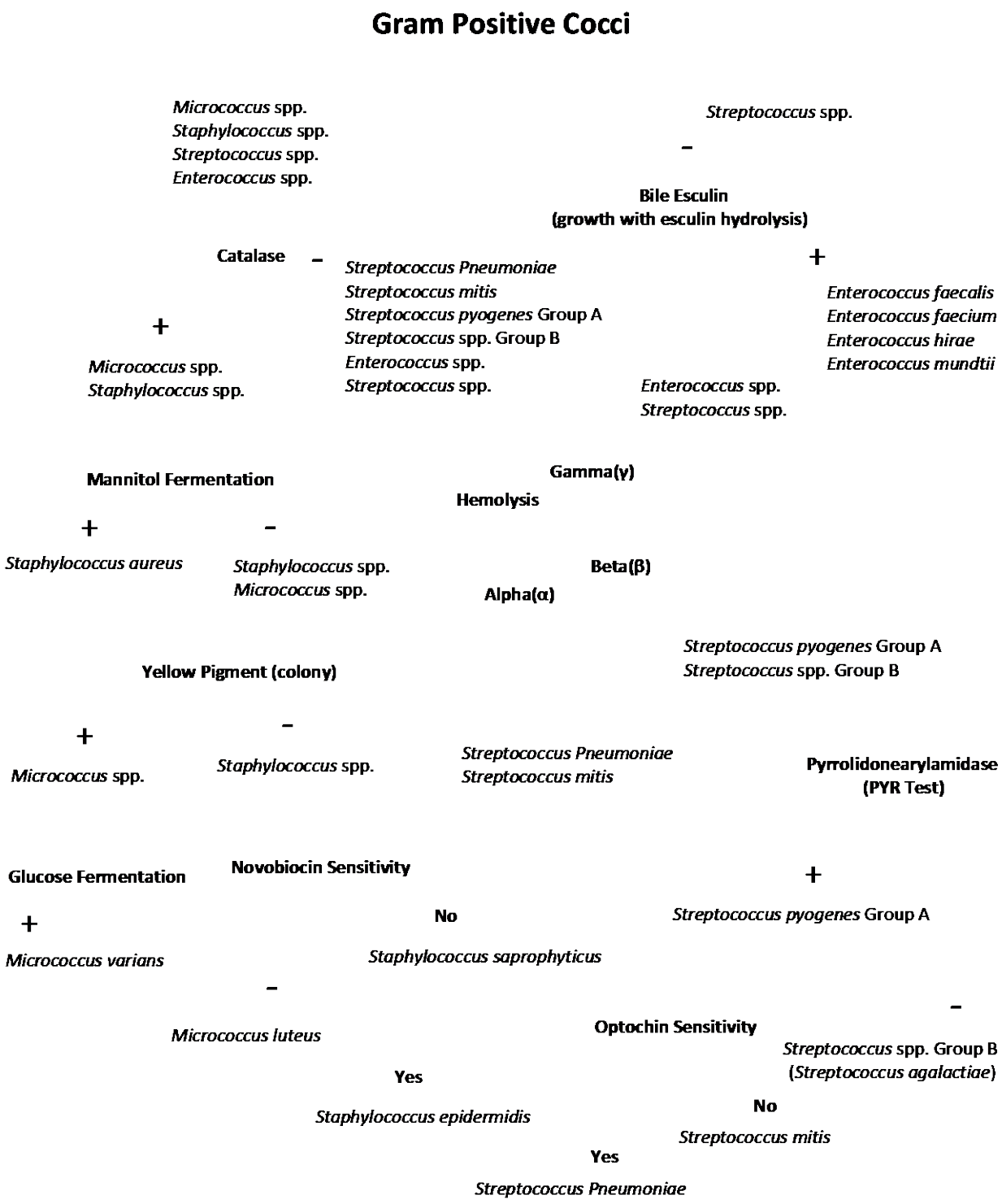
END OF WEEK 10

APPENDIX 1

Identification Flow Charts adapted from Bergey's Manual of Determinative Bacteriology



Gram Positive Cocci ID Flowchart



Gram Negative Rods ID Flowchart

Gram Negative Rods

Oxidase Test

\+ *Aeromonas, Pseudomonas, Vibrio*

\- *Enterobacteriaceae*

Glucose Fermentation

+ Acid — *Vibrio* spp., *Aeromonas* spp.

- Acid — *Pseudomonas* spp.

Na^+ Required for Growth

\+ *Vibrio* spp.

\- *Aeromonas* spp.

VP

\+
- *A. hydrophila*
- *A. salmonicida* (subsp. masoucida)
- *A. sobria* (no gas from glucose)
- *A. veronii*

\- Other *Aeromonas* spp.

Motility

\+
- *A. hydrophila*
- *A. veronii*

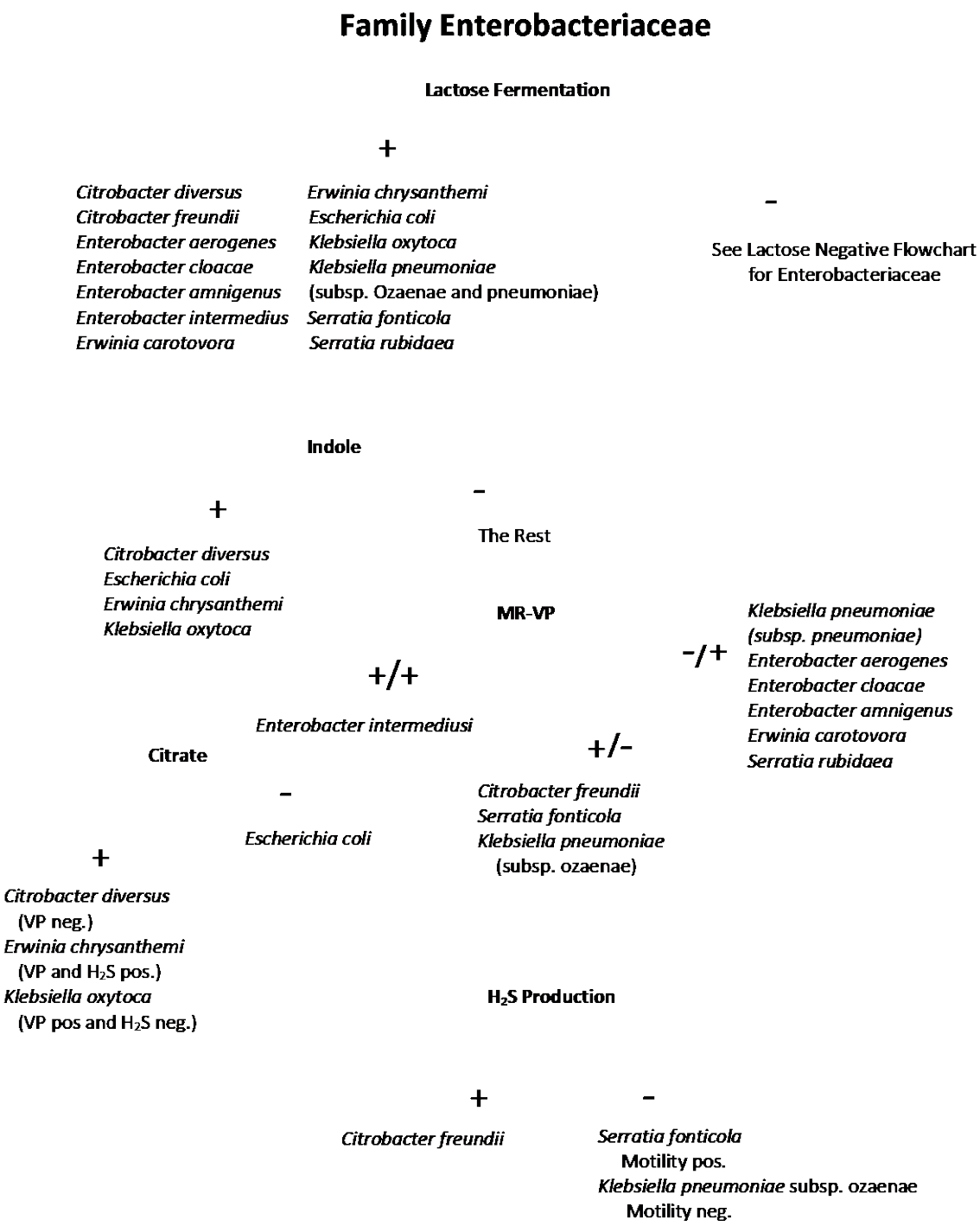
\- *A. salmonicida* (subsp. masoucida)

H_2S Production

\+ *A. hydrophila*

\- *A. veronii*

Family Enterobacteriaceae Lactose positive ID Flowchart



Family Enterobacteriaceae Lactose Negative ID Flowchart

Family Enterobacteriaceae Continued

Lactose Negative Flow Chart

Edwardsiella tarda
Erwinia cacticida
Morganella morganii
Proteus mirabilis
Proteus penneri
Proteus vulgaris
Providencia stuartii
Salmonella bongori
Salmonella enterica

Serratia marcescens
Serratia liquefaciens
Shigella spp. (*boydii, dysenteriae, flexneri*)
Shigella sonnei
Yersinia enterocolitica
Yersinia pestis
Yersinia pseudotuberculosis

\-

Erwinia cacticida
Proteus mirabilis
Proteus penneri
Salmonella bongori
Salmonella enterica
Serratia marcescens
Serratia liquefaciens
Shigella sonnei
Yersinia pestis
Yersinia pseudotuberculosis

Edwardsiella tarda
Morganella morganii
Proteus vulgaris
Providencia stuartii

\+

Indole Test

??

Please See Bergey's to ID:
Shigella spp. (*boydii, dysenteriae, flexneri*)
Yersinia enterocolitica

\+

Urease

\-

Erwinia cacticida
Salmonella bongori
Salmonella enterica
Serratia marcescens
Serratia liquefaciens
Shigella sonnei
Yersinia pestis

H_2S

\+

Edwardsiella tarda
Proteus vulgaris

\-

Morganella morganii
Providencia stuartii

Proteus mirabilis
Ornithine decarb. pos.
Proteus penneri
Ornithine decarb. neg., motile
Yersinia pseudotuberculosis
Ornithine decarb. neg., non-motile

Urease

\+

Proteus vulgaris

\-

Edwardsiella tarda

Motility

\+

Erwinia cacticida
(lysine decarb. Neg., the rest pos.)
Salmonella bongori
Salmonella enterica
Serratia marcescens
Serratia liquefaciens

\-

Shigella sonnei
Yersinia pestis

Salmonella bongori
KCN growth pos.
Salmonella enterica
KCN growth neg.
(It is this one)

\+

H_2S

\-

Serratia marcescens
Red pigment
Serratia liquefaciens
No Red pigment