Applied Biology APBI 403/SOIL 503

Field and laboratory Methods in Soil Science

(Biology Component)

Prepared by

Dr Sue Grayston

Faculty of Forestry & Faculty of Land & Food Systems

University of British Columbia

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Laboratory Sessions. Tuesdays 1-4pm MCML 102A (and *FSC 3401)

Date	Laboratory activity/analysis
Sept 6	Course overview, review of basic concepts of soil science
Sept 9	Sampling and sample preparation in Agriculture and Forestry (PacificSpiritPark and UBC farm)
Nov 4	Lab: Soil sampling Extraction of Macro- and Meso-fauna from Soil. (Berlese funnels, visual identification). Evaluation of soil faunal function using Bait-lamina test
Nov 18	Lab: Mycorrhizal fungi AM and ECM (% colonization and morphotyping). Nodules (Identification, N-fixation estimation using given acetylene reduction rates)
Nov 25	Lab: Soil Enzyme Assays (fluorometric microplate assay for phosphatase)* Demonstrations: Stable isotopes & molecular biology techniques

1. Introduction

1.1 Laboratory Classes

Class time will be utilized in a variety of activities, designed to provide an introduction to methods commonly used for soil biological analysis, and to reinforce concepts covered in lectures. Hands-on, demonstration, and discussion activities will be included.

Lab work can involve a variety of hazards. Students are required to read section 1.3. titled “Some Guidelines for Lab Operations” before commencing lab work.

1.2 Soil Biology Lab Report Objectives

In each lab period, you will be responsible for collecting data on sample(s) assigned to you. Also, in the Appendix you are given a table of biological analytical results (soil microbial biomass, enzyme activities and meso-faunal populations) for 12 organic and mineral soil samples collected pre- and post-harvest from the aggregated retention treatment of the STEMS experimental site on Vancouver Island. Samples were taken at three locations along a northerly transect at the centre, on the edge and at 30m from the edge of the aggregated retention patches into a clear-cut area.

Each member of the class will be required to write a final lab report on the soil biology component of this course using the results for these 12 soil samples. In the write-up students will need to choose one of the following comparisons between (i) mineral and organic soil horizons, (ii) pre- and post-harvest, or (iii) inside and outside aggregated retention plots.

The final lab report should be maximum 4 pages long, excluding all graphs and tables. Title of the report, student name, and "List of References" are not included in the four-page limit. Please use font size10 or 12 (preferable) with one and a half line spacing.

The final lab report should consist of the following sections: introduction, objective(s), material and methods, results and discussion, conclusions, and list of references.

1.3 Some Guidelines for Lab Operations

1.3.1 Safety

1. Lab coats and close-toed shoes MUST be worn at all times.
2. Do not eat or drink in the laboratory. Keep pens, pencils, fingers out of the mouth.
3. Some microorganisms are potential sources of infection under the correct conditions. Great care should be taken in handling cultures, slides and all material that has been in contact with living microorganisms.
4. Safety glasses, eye-shields, safety shields, respirators, gloves etc. should be used where appropriate (e.g. splash hazards, dust, naked flames, covering cuts etc.). You should make sure you know where this protection equipment is located.
5. Never work alone when significant hazards are involved; especially when working with microorganisms or inflammable materials, and chemicals.
6. Do not use your mouth to pipette soil solutions or chemicals, use the bi-pumps provided.
7. Do not lift bottles by neck only; support with hand below.

8. All spills should be cleaned up immediately.
8. All operations involving the risk of irritant or toxic vapor should be carried out in fume hood with adequate ventilation.
9. Know the locations of the nearest fire extinguishers, safety shower and first aid kit.
10. If you get an electrical shock from any equipment, report it, and do not use again until checked out by qualified person. Keep all electrical equipment dry.
11. Broken and chipped glassware is a hazard. Report breakages and dispose of broken glass only in the labeled receptacle provided for this purpose.
13. Exercise caution in moving around the lab, especially in crowded areas (e.g. near sink or fume hood).
14. Department policy is to prohibit use of perchloric acid ($HClO_4$) in the Department. (Even with satisfactory facilities, perchloric acid should never be used without thorough briefing from someone familiar with the hazards involved.)
15. Report ALL ACCIDENTS (e.g. spilled cultures, cuts or abrasions). Even apparently minor ones can develop serious side effects later on. In the case of eye injury in particular, immediate treatment is essential.
16. In case of fire, clear the area of personnel and call the fire department at 911. If no phone is available, pull the nearest alarm.
18. In general, make sure you understand the hazards involved before starting any lab operations. If in doubt, ask!
19. Wash your hands thoroughly immediately before leaving the lab. This applies to leaving the lab for any purpose DURING practicals too.

1.3.2 General

1. Label all solutions, plates and extracts clearly (contents, date, initials).
2. Dispose of unwanted samples, solutions etc. promptly and in a safe manner.
3. After using chemicals or solutions, make sure lids/stoppers are securely on, and return container to proper location (so others can find it; and rely on it).
4. Observe all possible cleanliness and neatness in the care of apparatus, microscopes and benches. When you leave the lab your bench should be clean and tidy. Microscope slides and cover slips should be discarded into the jars of disinfectant provided. Used pipettes should be placed, tip down, in the containers of disinfectant provided.
Test tubes must be kept upright in the test tube racks at all times.
5. Clean glassware as soon as possible after use. It is easier to get things really clean, if residues have not been allowed to dry on glass surfaces, especially with volumetric flasks, pipettes and burettes or other equipment with inaccessible regions.
6. Use volumetric flasks only as measuring tools, not as storage containers. They are too valuable for the latter use.
7. Soil samples should only be discarded in bins, not in the sink or garbage. Dispose of used petri dishes and microplates into the special autoclave bags provided.

8. Instruments should only be used after adequate instruction in their proper use. Misuse of instruments, apart from being expensive, interferes with the work of other students and staff.
9. Become familiar with resources on Lab Safety in the Department Reading Room (McML room #124), including MSDS and WHMIS material.

1.3.3 Class Lab Exercises

1. Adequate preparation is essential for good analytical work. The notes for the next laboratory session MUST BE READ before coming to class.
2. Record all your observations and results in an organized manner.
3. Tabulate results where possible (plan this ahead). Efficient systems of recording and manipulating data will avoid confusion and save time.
4. While students are expected to develop some ability to solve their own analytical problems, do not hesitate to ask questions.

2. Sampling of Soils

Physiochemical soil properties e.g. moisture, pH and nutrient concentrations vary spatially and temporally in forest ecosystems. Spatial variation can occur in two dimensions: vertical and horizontal. Horizontal variation in nutrient levels is high, often exceeding the annual temporal variation, even at scales as small as a few metres. This mosaic of fertility levels may be responsible for creating and maintaining diversity of plant species. Plants may also create variation because different plants have different nutrient demands and produce litter of differing quality. Plants also influence the composition and biomass of microorganisms as well as the rate of microbial processes. Spatial variations are autocorrelated (samples closer together are more similar). Therefore, sampling design is important, we take random samples (minimum four per location) and composite to represent one replication. We take ten replicates within a treatment area. It is very important to record other site details, slope, aspect, elevation, BEC zone and vegetation, classify humus forms and soil, record date, environmental conditions. Labeling of samples is vital. Sampling approach differs for extraction of soil macrofauna, mesofauna and microbes. Soil microbial analysis, ten cores extending 10 cm below the lower boundary of the organic horizon removed randomly from within 0.25 m^2 using a 3 cm diameter stainless-steel corer. Forest floor and mineral horizons are separated in the field and the samples from the ten cores composited in a plastic bag. Soil meso-faunal analysis, four soil cores (3 cm diameter) taken through the entire depth of the forest floor (LFH layers) and into the first 3 cm of mineral soil at the same locations. Forest floor and mineral portions of the core separated in the field, and packed in separate plastic bags. Forest floor portion of the core carefully wrapped to maintain structural integrity. Soil macro-faunal analysis, blocks (20 cm x 20 cm x ~6 cm) collected at the same locations used for the other sampling.

3. Preparation of Soils for Analysis

All soil samples taken for faunal analyses are kept cool during transportation and stored at 4° C in the lab before being extracted. Microbial samples are placed on ice in field, refrigerated within five hours, transported to lab within 2 days. Soils are sieved through a 2-mm mesh immediately on return to the lab, to remove roots and stones. Sub-samples stored at -20 ºC for chemical analysis, enzyme assays and 4° C for culturing, counts, freeze-dried for PLFA. Moisture content and bulk density measurements of the soils are also vital. Gravimetric moisture content is measured by weighing soil samples before and after oven-drying at 105 ºC for 48 hours.

4. Extraction of Macro and Meso-fauna from Soil and assessment of their activity

Objectives: To isolate, observe and quantify soil macro- and meso-fauna. To calculate species diversity, richness and evenness. To evaluate faunal activity in soil using the Bait-lamina test.

- Collect and extract macro- and meso-fauna from soil samples
- Use hand-sorting to extract macro-fauna from soil
- Identify macro-fauna using keys
- Use Berlese-Tullgren funnel to extract meso-fauna from soil
- Incubate funnels for 1 week under lights
- Identify meso-fauna using binocular microscope (will be week 2)
- Calculate meso-faunal species richness, diversity and evenness
- Incubate Bait-lamina strips in soil samples
- Evaluate feeding activity (Bait eaten vs not eaten) of soil fauna after 7 days

4.1. Background

Soil macrofaunal groups include earthworms, millipedes,centipedes, ants, Coleoptera (adults and larvae), Isopoda, spiders, slugs, snails,termites, Dermaptera, Lepidoptera larvae and Diptera larvae (see photos in theAnnexe - Identification Key on Soil Macrofauna).In terms of their abundance and their soil forming roles, earthworms,termites and ants are the most important macrofaunacomponents of soils and the importance of their activities has caused them to becalled “ecosystem engineers”.They burrow and are important in mixing the soil– known as bioturbation. Macroarthropods andMollusca are constant inhabitants of litter and, toa lesser extent, of soils, but they have generallymore specific ecological roles. Thus, most live in the litter or in the upper few centimeters of soil;saprophagous arthropods play a major role in thebreakdown of surface litter.

Soil meso-fauna are fauna of a body size within the range of 200 pm to 1 cm and include Acarina (mites), Collembola (springtails), Enchytraeidae (pot-worms), pauropods, tardigrades.

Most soil animals occur in the top 30 cm of soil, although some also occur atdepth. Soil animals may move to lower soil layers when conditions at the surface areharsh. Most soil animals occur in the surface layer because this layer contains themost food (C and nutrients) in the form of organic matter and other organisms.In both forest and agricultural systems, soil organisms perform vital functionsin the soil. The interactions among organisms enhance many of these functions,which are often controlled by the enormous amount of organisms in soils. Thesefunctions range from physical effects, such as the regulation of soil structureand edaphic (in soil) water regimes, to chemical and biological processes suchas degradation of pollutants,

decomposition, nutrient cycling, greenhouse gas emission, carbon sequestration, plant protection and growth enhancement orsuppression.

To reduce the huge complexity of organisms that live in the soil, a division ofsoil organisms into functional groups has been proposed.A functional group consists of a group of organisms that have the samefunction and similar impact on soil. The functions that soil organisms carry out dependlargely on the efficiency of their digestive systems (which themselves depend ontheir interactions with soil microorganisms, e.g. bacteria) and on the occurrenceand abundance of the biological structures that they produce in the soil. Using thesetwo criteria, three large functional groups of invertebrates can be distinguished:micropredators, litter transformers, and ecosystem engineers. The micropredator group contains the smallest invertebrates, protozoa andnematodes. They do not produce organo-mineral structures,and their principal effect is to stimulate the mineralization of soil organic matter(SOM).In the litter-transformer group, mesofauna and some macrofauna organisms are involved in litter decomposition. When these invertebratesre-ingest their excretions, which serve as incubators for bacteria, they assimilate metabolites liberated by microbial actions.The “ecological engineers” or “ecosystem engineers” arethose organisms that produce physical structures through which they can modifythe availability or accessibility of a resource for other organisms. Among theinnumerable life forms that inhabit soils, only a small number of macro-invertebrates(earthworms, termites and ants) are distinguished by their capacity to excavate soiland produce a wide variety of organo-mineral structures, such as excretions, nests,mounds, macropores, galleries and caverns. Their structures have been describedas “biogenic structures”. Their activities and biogenic structurescan modify the abundance or structure of their communities. The functional role of these structures is thought to be important because they represent sites where certain pedological processes occur: stimulation ofmicrobial activity; formation of soil structure; SOM dynamics; and exchange ofwater and gases.

The biodiversity of soil organisms is important in maintaining beneficial ecosystem functions. The higher the biodiversity of a community, the more resilient the ecosystem will likely be to perturbation. Low levels of biodiversity can weaken an ecosystem’s natural cycles, leaving it susceptible to extreme events. Biodiversity is a measure of the number of species in a given habitat (species richness), as well as how many individuals of each species are present (species abundance) and how evenly these individuals are distributed (species evenness). It can be calculated with the Shannon Index using the proportions of different species found in an ecosystem. Values tend to range between 1.5 and 3.5. The higher the index value, the more diverse the community.

4.2. Materials

Burlese-Tullgren funnels

Beaker containing 70% ethanol

Petri dishes for examining meso-fauna

Bait-lamina test strips

Large beaker to incubate test strips in soil samples

4.3. Methods

Extraction of soil macrofauna

1. At each plot, place 20 cm x 20cmwooden frame on forest floor.

2. Using knife and mallet, cut soil block until just into mineral soil.

3. Extract block and place entire sample in Tupperware box and label.

4. Macro-fauna are hand-sorted from the soil blocks collected.

5. Macro-fauna are placed in petri dishes and identified using the keys provided.

6. Count the number of individuals of each group present in each sample. Write down the results in a table.

Extraction of soil mesofauna

1. At each plot, remove 5 cm diameter cores through entire forest floor and top 3cm mineral soil.

2. Extract core and place entire sample in collection bag and label.

3. For each sample, measure 50-60mL of 70% ethanol in screw cap specimen jars.

4. Place an empty jar beneath Burlese-Tullgren funnels.

5. Place litter sample into Burlese-Tullgren funnel on top of screen and mesh (9” diameter ¼” screen, 8” diameter finer screen, two layers of 8” diameter mesh or cheesecloth).

6. Shake and hit funnel so any loose soil falls through. Make sure no soil is stuck at the bottom by tapping against it near the hole. Place any fallen soil back into funnel.

7. Replace empty jar with jar of ethanol.

8. Place lamp on top of funnel.

9. Let sit for 7 days.

10. Remove jar of ethanol and store until ready for invertebrate identification.

11. Etch a 1cm x 1cm grid into the bottom of petri dish.

Perform the invertebrate identification WEEK 2

12. Transfer an equal portion of the extraction to each of 4 petri dishes.

13. Make sure to rinse the jar so the entire specimen is transferred.

14. Each member of the group will identify the organisms on each Petri dish.

15. Using a dissecting microscope and provided keys, observe the contents of the petri dish starting with the upper left-hand corner of the grid moving right, then down a row moving left, and so on.

16. Record any observations of invertebrates, including millipedes, springtails, and mites using the given tally sheet and invertebrate key for identification.

17. Use the tally sheet to calculate the proportion of each species found in your sample. Making a chart may be helpful.

18. Compile data from replicate plates and compute the following indices (below).

Evaluate soil faunal activity using Bait-lamina test strips

19. Fill large beaker provided with sample of soil.

20. Insert 4 x 16 bait-lamina strips vertically into the soil.

21. Incubate for 7 days in the lab.

22. Evaluate bait consumption after washing the strips carefully under flowing tap water and then examining on a lighted bench, where differentiation is made only between "bait eaten" (means light falls through the bait) and "bait not eaten".

23. Calculate % feeding activity per 16 holes (1 strip). Compare results from different sites.

4.4. Questions

1. Calculate faunal richness by summing the total of organism groups.

2. Calculate faunal species diversity (H') using the Shannon Index:

$H' = - \Sigma [p_i * \ln (p_i)]$ p_i is the proportion of all observed organism groups

3. Using the H' from the other groups, calculate evenness of the communities:

$E = H' / H'_{max}$

4. Calculate % activity fauna form bait strips and compare with results from other groups soils.

* Enter your summary data in the shared soil fauna file on connect

Example Table below

	Species	Abundance	Proportion (p_i)	$- p_i * \ln (p_i)$
	Mites	50	0.5	0.347
	Springtails	30	0.3	0.361
	Isopods	10	0.1	0.230
	Millipedes	9	0.09	0.217
	Spider	1	0.01	0.046
Total	5	100	1.00	1.201 (H')

4.5. Discussion

1. What is the importance of invertebrate diversity in soil?

2. What are some important ecosystem functions of invertebrates?

3. Compare your results of invertebrate diversity with that of other groups.

4. Why do some ecosystems contain more invertebrate diversity than others?

5. Why do the different ecosystems have varying faunal activity?

Marks: Questions/Calculations 14 marks; Discussion questions 10 marks. Total 24 points

4.6. References

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Stork, N. E. &Eggleton, P. 1992. Invertebrates as determinants and indicators of soil quality. *Am. J. Alt. Agr.*, 7(1–2): 38–47.

Swift, M. J., Heal, O. W. & Anderson, J. M. 1979. *Decomposition in Terrestrial Ecosystems*. Blackwell Scientific, Oxford.

5. Assessing fundamental symbiotic relationships between plants and soil microorganisms (Identify characteristics of Arbuscular and Ectomycorrhizal fungi and estimate % colonization of roots by staining and visual estimation (AM) and morphology (EM) using microscopy and photographic keys; identify root nodules and estimate N_2 fixation rates).

Objectives: To observe, characterize and enumerate % colonization of plant roots by mycorrhizae using staining, microscopy techniques and morphotyping. To identify root nodules, enumerate and estimate N_2 fixation rates.

- Clear and stain root samples for AM assessment
- Estimate % colonization of roots by visualization under dissecting microscope
- Retrieve fine roots of tree species for EM assessment
- View under the microscope and describe the morphotypes you see using the on-line key, and estimate the % colonization of roots by different fungi
- Count nodules on roots, dissect nodules to determine if nodules are active, weigh total nodule mass per plant
- Use biological nitrogen fixation rate per nodule (given) to calculate the overall rate of BNF per plant

5.1. Background

Microorganisms are vital components of soil. They are responsible for decomposition and nutrient cycling processes, they produce and consume gases governing the global climate and they have many biotechnological applications (e.g. waste water treatment, bioremediation, biocontrol, antibiotic and other medicinal products, food and alcohol). Soils contain billions of bacteria and millions of fungal propagules per gram. Some of these microorganisms are free-living in the soil growing on litter material for their sustenance, others are obligate or facultative symbionts with plants. Examples of beneficial symbiotic associations between plant roots and microorganisms include mycorrhizae which are a mutualistic association between fungi and plants. The fungus receives its carbon for growth from the host plant and in return supplies the plant with increased water and nutrient uptake capacity. There are 3 main types of mycorrhizae; arbuscular (AM), which are formed on the majority of herbaceous plants; ericoid which are formed on ericaceous plants such as salal and ectomycorrhizae (EM) which are formed on the majority of temperate tree species. Though the advent of molecular methods to identify soil microorganisms has replaced many of the traditional techniques for studying these organisms (and you will learn about these techniques in the molecular methods demonstration lab in lab 3), the isolation of these microorganisms from the complex mixed populations in soil is still

essential for physiological studies. In addition, in many cases, rapid, relatively inexpensive methods are preferred for first-stage screening and assessment of the effects of, for example, different reclamation treatments, different cover crops, pollutant contamination, on these symbioses. Methods for studying mycorrhizae are numerous; one of the most common, first-stage, assessments of mycorrhizae is to assess % colonization of roots using root-clearing, staining and visual estimation of % infection for arbuscular mycorrhizae (as the majority of the fungus in AM is internal within the root cortical cells) and morphotyping and visual estimation of % infection for ectomycorrhizae (as the majority of the fungus in EM is external, forming a mantle on the fine root tips) using microscopy and photographic keys. The morphotyping of ectomycorrhizae also enables an initial identification of the mycorrhizal fungus partner in the symbiosis as there are many different growth forms and colours characteristic of these fungi.

Another common and extremely important symbiosis is that between legumes and *Rhizobium* or *Bradyrhizobium* species of bacteria and that between alder trees and *Frankia* species of actinomycetes. Rhizobia and Frankia infect root hairs of the leguminous plants and produce nodules. The nodules become the home for the microorganisms where they obtain energy from the host plant and in return take free nitrogen from the soil air and process it into combined nitrogen through biological nitrogen fixation (BNF). This is the process whereby atmospheric nitrogen (N=N) is reduced to ammonium in the presence of the nitrogenase enzyme. Nitrogenase is an oxygen sensitive enzyme. The low oxygen tension condition is realized through synthesis of leghaemoglobin (in *Rhizobium* legume). Leghaemoglobin is a macromolecule synthesized by both symbiotic partners, the rhizobia and the host plant. *Rhizobium* synthesizes the heme portion, and the plant the globine. Like human haemoglobin, leghaemoglobin fixes O_2. It is responsible for the red or brown color of active (i.e., N_2-fixing) nodules. Non-N_2-fixing nodules have a white nodule content, or a green content when the globine has degenerated. Plants receive the fixed N from nodules and produce food and forage protein and can be used to enrich soils with N for following crops. N_2-fixing systems can thrive in soils poor in N, explaining the ability of alder to colonize highly disturbed ecosystems, such as abandoned logging roads and quarries.

Nodules are easy to identify on legume and alder roots. They are generally assessed by enumerating nodule number and mass and then nitrogenase activity using the acetylene reduction technique. Nitrogenase not only catalyzes the reduction of atmospheric N_2 to NH_3, but can also reduce acetylene (C_2H_4). The acetylene reduction assay (ARA) is carried out on detached nodules, de-topped roots, or whole plants in a closed vessel containing 10% acetylene. A gas chromatograph is used to determine the amount of ethylene formed. Data are usually expressed as nanomoles or micromoles of ethylene produced per hour per plant or per weight unit of nodules. The acetylene reduction assay provides an instant measure of nitrogenase activity (but not necessarily of N_2 fixed) under the experimental conditions. For the purposes of this laboratory you will be given rates of BNF per nodule and then you can calculate rates of BNF per plant for your sites.

5.2. Materials

Petri dishes

Beakers

Fine forceps

Razor blades/scalpels

10% KOH

Schaeffer black ink

White vinegar

Bunsen burner

Dissecting microscope

Lamps

Pipettes

Filter paper

Weighing balance

Microscope camera (digital eyepiece)

5.3. Methods

Sampling of soils and roots

Soils and roots are sampled for mycorrhizal analysis using the core method outlined in section 2 above.

For root nodule assessment whole plants and their roots are excavated from soil (for herbaceous plants –legumes and tree seedlings), for larger trees roots are excavated and representative roots and nodules removed.

Clearing and staining of herbaceous roots for AM assessment

Roots from at least five replicate plants per treatment should be stained. Root length is measured and then roots are cut into 1 cm segments and cleared of tannins by boiling in 10% (wt/vol) KOH for 3 minutes and then rinsed several times with tap water. To stain cleared roots are boiled for 3 min in a 5% Shaeffer black ink-vinegar solution with pure white household vinegar (5% acetic acid). Roots are destained by rinsing in tap water (acidified with a few drops of vinegar) for 3

minutes. The stained root pieces are then spread out evenly on a petri dish and observed using a dissecting microscope. By moving the dish and observing the whole sample an overall estimate of the % cortex infected can be made. Replication to find the SD of the method is obtained by rearranging the same root sample and making another visual estimate.

Visualizing EM on fine roots of trees

Cleaning Procedure and ECM Extraction from Soil

Immerse the soil core in water and soak carefully in water until saturated. Wash the roots gently with pipettes to limit damage to the ectomycorrhizas then place cleaned roots (<1 mm diameter) in a Petri dish with filter paper soaked with water **(**Petri dishes with filter paper prevent color changes of ectomycorrhizas and hyphal growth). ECM tips can be stored in the refrigerator up to seven days after sampling.

ECM Morphology

Observe ECM root tips in water under a dissecting microscope (6×, 12×, 25×) using a black background and lamps of daylight quality. Isolate morphotypes by morphology, i.e., color, ramification type, systems, size and texture, presence of emanating hyphae, cystidia, rhizomorphs, and/or sclerotia (see B.C. ectomycorrhizal research website and Agerer and Rambold 2004–2007). (3) Take photos of mycorrhizal systems, maintaining the black background and lamps of daylight quality (Agerer 1991). Estimate % infection of roots by different mycorrhizal fungi. Estimate ECM abundance and richness.

Nodules

Wash roots gently with pipettes to minimize damage to nodules. Place nodules in petri dish and count nodule numbers. Calculate nodule mass by weighing nodules. Using a scalpel dissect nodules and determine the % active (brown/red) versus inactive (white/green) nodules. Using a BNF rate of 10 nmol per hour per nodule calculate the overall BNF per plant per day.

5.4. Calculations

% infection
Number of roots with AM or EM infection/Number of uninfected roots x 100/1

5.5. Questions

Mycorrhizae

1. Calculate % infection of roots by AM fungi.
2. Were there quantitative differences in % AM infection in your different plant samples? Explain why you think this is the case.
3. Illustrate what you saw under the microscope with drawings/photos and labelling of key features.
4. Calculate % infection of woody roots by ECM fungi. Note differences in color, ramification type, systems, size and texture, presence of emanating hyphae, cystidia, rhizomorphs, and/or sclerotia.
5. Were there different ECM fungi on your root samples? Explain why you think this is the case.
6. Illustrate what you saw under the microscope with drawings/photos.
7. Did you manage to identify your main Ectomycorrhizae with the keys?

Nodules

1. Count the total number of nodules for each plant.
2. Calculate nodule mass by weighing nodules.
3. Using a scalpel dissect nodules and determine the % active (brown/red) versus inactive (white/green) nodules.
4. Using a biological nitrogen fixation (BNF) rate of 10 nmol per hour per nodule calculate the overall BNF per plant per day.
5. Were there qualitative and quantitative differences in nodule numbers and mass in your different plant samples? Explain why you think this is the case.
6. Did some samples contain more active nodules than others? Why might this be the case?

5.6. Discussion

1. What are the advantages/disadvantages, strengths/weaknesses of the methods used in this laboratory?
2. Why are mycorrhizae important?
3. Do you think mycorrhizal diversity is important?
4. Why might there be more ECM diversity in some samples than others (what factors might influence diversity)?
5. What factors might affect rates of BNF?
   Marks Mycorrhizal Qs 6 marks; Nodule Qs 8 marks; Discussion 10 marks. Total 24Marks.

5.7. References

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6. Soil Enzyme assays (fluorometric microplate assay for phosphatase)*

Objective: ***To analyze the activity of enzymes associated with soil microorganisms that are important in nutrient cycling.***

• Extract extracellular enzymes from soils

• Perform microplate fluorometric enzyme assays for phosphatase activity.

• Analyze MUB concentrations fluorometrically to give an estimate of phosphatase activity

6.1. Background

Enzymes are proteins with substrate-specific activity that can quickly and easily be measured in the soil. Enzymes naturally originate from plant, animal, and microbial sources, although microbes are the major contributors to soil enzyme activity. They may be present in the soil via excretion from living organisms or leakage from dead cells, and may be free to react with substrates, or be bound to soil components. The activities of extracellular soil enzymes produced by soil microorganisms play an important role in nutrient cycling because they catalyse the breakdown of complex organic matter into nutrients that are available for plant uptake. Enzyme activity is affected by soil physico-chemical characteristics such as temperature, moisture, and substrate availability, as well as microbial biomass and the presence of vegetation. Soil enzyme activity levels directly represent potential degradative ability of the soil community. Enzyme activity has been shown to respond quickly to environmental disturbances, and it is easy to measure. The most important enzymes in the forest soil are those that degrade the most abundant biopolymers, the organic compounds lignin and cellulose, as well as those that cycle P and N. β-1, 4-glucosidase is an enzyme produced by bacteria, fungi and protozoa and is involved in cellulose degradation. Organic forms of nitrogen, a component of the ubiquitous chitin polymer, are mineralised by chitinase. N-acetyl-β-glucosaminidase (chitinase) is naturally produced by bacteria, fungi and plants. Acid phosphatase, produced by plant roots and soil microorganisms, catalyses the breakdown of organic P and releases plant available inorganic P. There is a strong relationship between P availability and P mineralization. Phosphorus is processed biochemically as it is needed and phosphatase enzyme production is correlated with microbial demand for P. Phosphatase activity has been shown to be negatively correlated with P concentration in the soil. Peroxidase and phenol oxidase are oxidative enzymes produced by white-rot fungi that play an important role in lignin degradation. These enzymes are classified by what they use as an oxidant; phenol oxidase uses oxygen and peroxidase uses hydrogen peroxide. Phenol oxidase activity is correlated with fungal biomass and organic matter degradation in soil, which shows that enzyme activity can be an indicator of microbial habitat and activity.

Enzyme bioassays are used to obtain potential activity rates of specific extra-cellular enzymes in the soil samples when incubated with a synthetic substrate at a consistent pH and temperature. The microplate enzyme bioassay methods of Marx *et al.* (2001) and Sinsabaugh *et al.* (2003) were used as a basis for developing colorimetric and fluorometric enzyme assay protocols modified for our laboratory. The determination of enzyme activity using 4-methylumbelliferyl (MUB) substrates is a highly sensitive technique. Colorimetric methods for studying soil enzymes are less sensitive than fluorometric methods, though the cost of fluorometric substrates is greater. Moreover, colorimetric methods suffer from an interference problem caused by highly coloured phenolic compounds in the dissolved organic carbon of organic soil and forest floors, which cause high background absorbances. However, currently there are no fluorometric substrates for oxidative enzymes. As a consequence fluorogenic (methylumbelliferyl) substrates are preferred for analysis of hydrolytic enzymes and colorimetric for oxidative enzymes. Fluorogenic substrates have been widely adopted due to the great sensitivity of the fluorimetric detection procedure. The technique uses the compound methylumbelliferone (MUB) which fluoresces at 450 nm emission 330 nm excitation. A range of compounds can be chemically attached to MUB causing the loss of the fluorescent property. However, upon enzymichydrolysis of the bond between MUB and the attached compound, the MUB is liberated, and its fluorescence can be readily measured and related to the activity of the hydrolytic enzyme.

L-3, 4-dihydroxyphenylalanine (L-DOPA) is the colorimetric substrate used to test for phenol oxidase and peroxidase activity. Oxidation of DOPA by these enzymes results in the production of the chromophore, dopachrome, which has an absorbance maxima at 460 nm.

6.2. Materials

a. Fluorometric assay (solutions and buffer prepared for you)

i) Autoclave Nalgene bottles and pipette tips.

ii) Prepare substrate stock solutions (100 ml of 200 µM solution in sterile water). Substrate powders are kept at -20 ºC. *Solutions are stable for weeks if not contaminated, keep at 4 ºC*. **Make phosphate solution fresh each time. BE CAREFUL NOT TO CONTAMINATE!**

Substrate	**Amount (g) in 100 ml dH_20**
4-MUB-β-D-cellobioside	0.01
4-MUB-β-D-galactoside	0.0068
4-MUB-β-D-glucoside	0.0068

4-MUB-β-D-glucuronide	0.007
4-MUB-β-D-mannopyranoside	0.0068
4-MUB-β-D-xyloside	0.0062
4-MUB-N-acetyl-β-glucosaminide (NAG)	0.0076
4-MUB-phosphate	0.0051
4-MUB-sulfate	0.0059

iii) Prepare standard 10 mM***stock solution*** of 4-methylumbelliferone in methanol, keep at -20 ºC. **Dilute to 10 µM in distilled water – *working solution* - keep at 4 ºC.**

iv) Prepare sodium acetate buffer. Initially make up 500mM. If making up 1.5 L – add 102.06 g of sodium acetate powder into a glass beaker. Place beaker on stir plate with stir bar and add approx 400ml dH20. Keep stirring until powder has dissolved – add more water if necessary. When dissolved calibrate pH meter (pH 7 and pH4) and then add glacial acetic acid until pH drops to 5. Transfer to a large flask and add dH20 to make up 1.5 L. Autoclave the buffer on P6 cycle. Keep at 4 ºC.
Then (when cooled) make up a bottle of 50mM working concentration buffer using dH20. Keep at 4 ºC.

b. Colourimetric assay (solution and buffer prepared for you)

1. Prepare DOPA solution: Make up 100ml of 25mM L-3, 4-dihydroxyphenylalanine (DOPA) in 50mM acetate buffer (pH 5.0). To make up 100ml of solution weigh out 0.493g of the DOPA into 100ml of the 50mM acetate buffer. Use a bit of heat (1-2 on the heat plate) and a stir bar to dissolve the DOPA. Keep all beakers and bottles covered in foil – the DOPA solution must be protected from light at all times. DOPA substrate can be stored in the fridge overnight as long as precipitate isn't visible.

2. Prepare sodium acetate buffer. Initially make up 500mM. If making up 1.5 L – add 102.06 g of sodium acetate powder into a glass beaker. Place beaker on stir plate with stir bar and add approx 400ml dH20. Keep stirring until powder has dissolved – add more water if necessary. When dissolved calibrate pH meter (pH 7 and pH4) and then add glacial acetic acid until pH drops to 5. Transfer to a large flask and add dH20 to make up 1.5 L. Autoclave the buffer on P6 cycle. Keep at 4 ºC. Then (when cooled) make up a bottle of 50mM working concentration buffer using dH20. Keep at 4 ºC.

6.3. Methods and Calculations

a. Phosphatase - Fluorometric Enzyme Assay

1. Prepare soil samples (aim for maximum of 50% quench).
    - From frozen – grind slightly more soil than is needed in a pestle and mortar for 20 seconds.
    - Rinse pestle and mortar with 70% ethanol and then with distilled H20.
    - Weigh 0.1 g of the ground forest floor/humus or mineral soil into a 125 ml screw cap Nalgene bottle.
    - Add 50 ml of 50 mM acetate buffer (pH 5.0) and approx. 30 glass beads (pre-wash the glass beads in ethanol, rinse thoroughly in dH20 and drain).
    - Shake bottles for one hour on high.
    - Add another 50 ml of buffer to the bottles.
    - Keep solutions at 4 ºC until ready to use (preferably use immediately – but not longer than overnight).
2. Label plates using green tape. Add buffer and MUB standard to the plates – keep at 4 ºC until ready to add soil and substrate.
3. Pipette reagents into black microplates;

Enzyme name and plate replicate #	
Std	200ul buffer + 50ul 4-MUB std
Sub	200ul buffer + 50ul sub (4-MUB-phosphate)
S1	Your test soil # 1 200 ul soil suspension + 50ul substrate (4-MUB-phosphate)
S1	Your test soil # 1 200 ul soil suspension + 50ul substrate (4-MUB-phosphate)
S2	Your test soil # 2 200 ul soil suspension + 50ul substrate (4-MUB-phosphate)
S2	Your test soil # 2 200 ul soil suspension + 50ul substrate (4-MUB-phosphate)
Q1	200 ul #1 soil suspension + 50ul 4-MUB standard
SB1	200 ul #1 soil suspension + 50 ul buffer
Q2	200 ul #2 soil suspension + 50ul 4-MUB standard
SB2	200 ul #2 soil suspension + 50 ul buffer
…	Empty
BB	250ul buffer

Sample plate outline for fluorimetric enzyme bioassay. Standard (Std), Sample (S), Substrate (Sub), Quench (Q), Soil Buffer (SB), Background Buffer (BB).

4. Add soil suspension, shake bottle thoroughly and re-suspend sample in trough each time (pipette up and down). **Use a different trough for each soil sample and different pipette tips.**

5. Add 50 µl of substrate to appropriate wells and record time after addition.

6. Incubate plates at 20 ºC in the dark. Incubation time depends on substrate;
    - Phosphatase: 2 hours
    - B-glucosidase: 3 hours
    - NAGase: 3 hours
    - Sulfatase: 3 hours
    - Xylosidase: 4 hours
    - Galactosidase: 5 hours
    - Cellobiohydrolase: 7 hours

7. At the end of the incubation add 20 µl aliquot of 0.5 N NaOH to each well.

8. **Warm up microplatefluorimeter AND turn on lamp for 10 minutes prior to first reading**. In Cytofluor programme **s**et excitation at 360/40 nm and emission at 460/40 nm. Set gain to 50. Set mix time for 5 seconds. Set plate type to Costar. Export each plate reading separately as an Excel file.

9. Calculate activity as nmol of substrate converted per hour per gram of sample.

$$\text{Signal (nmol)} = \frac{F_{assay}}{Ec*Qc} - \frac{F_{sub}}{Ec} - \frac{F_{soil}}{Ec*Qc} + \frac{F_{buffer}}{Ec*Qc}$$

$$\text{Activity rate (nmol } h^{-1} g^{-1}) = \frac{\text{signal} * 100 \text{ ml}}{T_i * 0.2 \text{ ml} * 0.01 \text{ g}}$$

Ec (Emission coefficient) (fluor/nmol) = mean fluorescence of reference standard/0.5

Qc (Quench coefficient) = (mean fluorescence of quenched standard/mean of fluorescence of reference standard)

F_{assay}= mean fluorescence of assay wells

F_{sub} = mean fluorescence of substrate background wells

F_{soil} = mean fluorescence of soil background wells

F_{buffer} = mean fluorescence of buffer background wells

T_i = incubation interval (hr)

***If calculated activity rate is negative, then actual rate = 0.**

6.4. Questions

1. Calculate the activities of phosphatase in the organic and mineral soil horizons of the agricultural and forest soil samples.
2. Why do some soils have greater enzyme activities than others?
3. Why do the activities of phosphatase differ between different soil horizons?
4. Complete the following table for the different enzymes - listing the natural substrate of each enzyme, describe reaction details (what enzyme does, who produces the enzyme), class of enzyme (induced, repressed, adaptive, constitutive) and the products produced.

Name(s) of enzyme	Assay substrate	Natural substrate group	Reaction details	Class of enzyme	Product(s) of interest
Acid phosphomonoesterase	4-MUB-phosphate				
Acid phosphodiesterase	4-MUB-phosphate				
Alkalinephosphomonoesterase	4-MUB-phosphate				
Cellobiohydrolase	4-MUB-beta-D-cellobioside				
Beta-1,4-glucosidase	4-MUB-beta-D-glucoside				
Beta-1,4-xylosidase	4-MUB-beta-D-xyloside				
Beta-1,4-N-acetylglucosaminidase (NAG)	4-MUB-N-acetyl-beta-D-glucosaminide				
Phenol oxidase	L-3,4-Dihydroxyphenylalnine				
Peroxidase	L-3,4-Dihydroxyphenylalnine				
Aryl sulfatase	4-MUB-aryl-sulfatase				

Marks: Calculation of enzyme activities 8 marks; questions 6 marks; table 10 marks; Total 24 marks.

6.5. References

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SinsabaughRL 1994.Enzymic analysis of microbial pattern and process.Biology & Fertility of Soils 17, 69-74.

Sinsabaugh, R.L., Saiya-Cork, K., Long, T., Osgood, M.P., Neher, D.A., Zakd, D.R., Norby, R.J., 2003. Soil microbial activity in a *Liquidambar* plantation unresponsive to CO_2-driven increases in primary production. Applied Soil Ecology 24, 263-271.

7. Appendix

7.1. Data Table. Biological analytical results (soil microbial biomass, enzyme activities and meso-faunal populations) for 12 organic and mineral soil samples collected pre- and post-harvest from the aggregated retention treatment of the STEMS experimental site on Vancouver Island.

Location	Soil Horizon	Phosphatase (nmol h^{-1} g^{-1})		Glucosidase (nmol h^{-1} g^{-1})		N-Acetyl Glucosidase (nmol h^{-1} g^{-1})		Phenol oxidase (nmol h^{-1} g^{-1})		Mites (# m^{-2})		Collembola (# m^{-2})		Microbial biomass (nmol g^{-1})	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Centre	Organic	1067	1220	791	443	491	295	658	1240	280,000	270,000	81,000	100,000	184	215
Centre	Mineral	476	485	47	110	69	113	1525	1790	12,000	-	3,000	-	59	62
Edge	Organic	1084	1147	761	531	482	339	633	770	260,000	190,000	80,000	40,000	185	190
Edge	Mineral	564	494	61	125	84	104	1360	1655	15,000	-	2,500	-	57	54
30 m	Organic	1067	853	596	426	680	202	739	855	310,000	115,000	105,000	20,000	167	184
30 m	Mineral	639	406	72	175	131	157	1637	1155	20,000	-	3,500		45	49

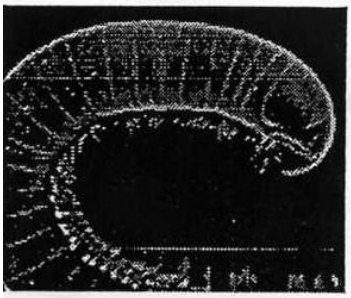
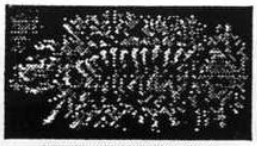
Samples were taken at three locations along a northerly transect at the centre, on the edge and at 30m from the edge of the aggregated retention patches into the clear-cut area.

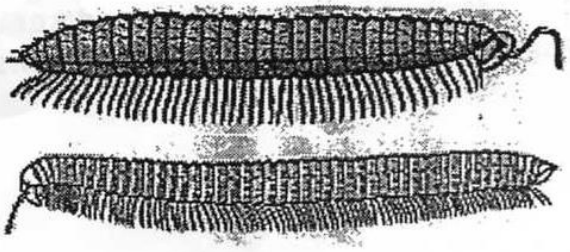
STUDENT KEY to B.C. FOREST SOIL ANIMALS.

1 (a) Antennae present. _____ 2
(b) Antennae absent. _____ 7

2 (a) *3 pair legs*; 3 fairly distinct body regions. _____ 11
(b) *7 or more pair legs*; head distinct from rest of body. _____ 3

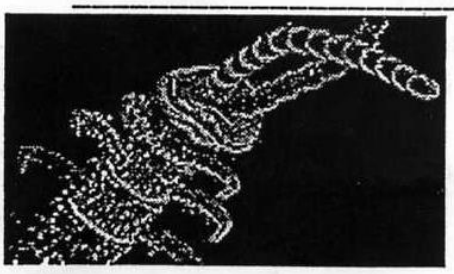
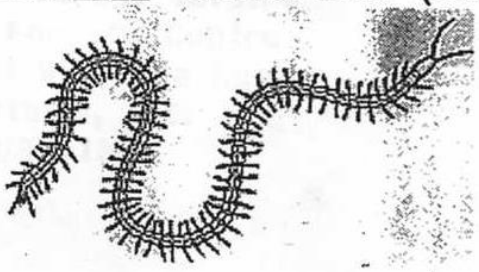
3 (a) *Legs arranged in double pairs*; most segments with 2 pair legs. _____ **DIPLODA (Millipedes)**



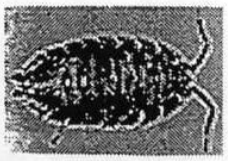
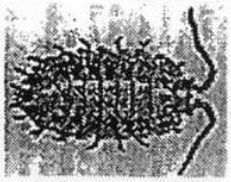
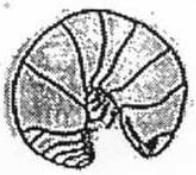
(b) *Legs arranged in single pairs,* usually 1 pair per segment. ____ 4

4 (a) *15 or more pair legs*; body flattened dorso-ventrally. _____ **CHILOPODA (Centipedes)**

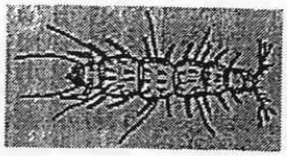
(b) *7-12 pair legs.* _____ 5

5 (a) *7 pair walking legs* ;1 pair per thorasic segment; appendages (uropods) on last body segment usually stick out; body oval, flattened & hard; usually dark; can curl. ___**CRUSTACEA: ISOPODA (Woodlice)**


(b) 9-12 pair legs; body usually elongate. _____ 6

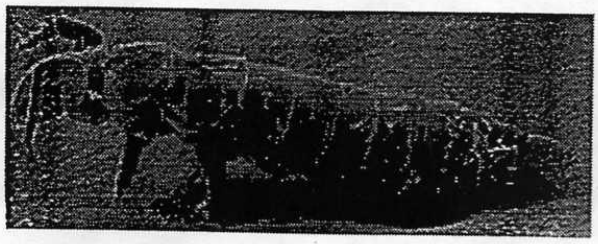
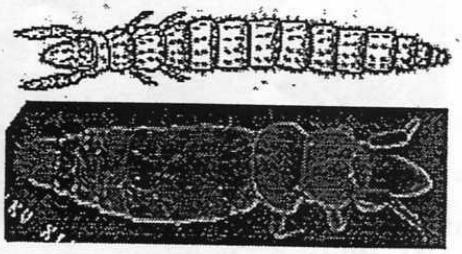
6 (a) *Antennae branched*; 12 segmented body; *9 pair functional legs* in adults; tactile bristles on sides;*small (few mm)* . ___ **PAUROPODA**

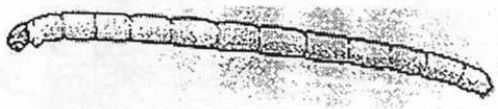
(b) *Antennae not branched* but many segmented & slender; 10-12 pair legs; 15-22 body segments; *larger (1-8 mm long).* ___ **SYMPHYLA**


7 (a) Body elongate & segmented; 3 pair of legs or without legs. _____ 8
(b) Body not elongate; may or may not be segmented; usually with 4 pair legs. _____ 9

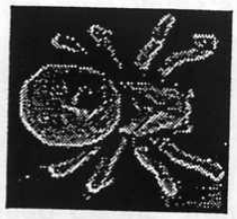
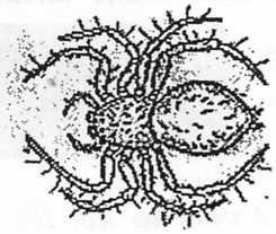
8 (a) Minute animals (*0.6-1.5 mm*); *3 pair of legs with the 1st pair directed anteriorly*; eyes absent; much of the mouthparts withdrawn into the head; whitish in color. _____ **PROTURA**

(b) Large animals; with or without legs; mouthparts either reduced to hooks (Diptera larvae) or well developed chelate types; eyes present or absent; usually brownish in color. _____ **INSECTA LARVAE**

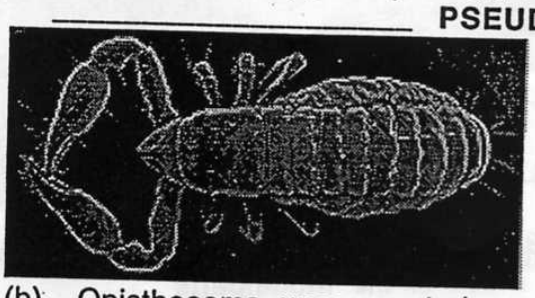



9 (a) *Body petiolate* (with a narrow "waist") _____ **ARANEIDA (Spiders)**

(b) Body not petiolate. _____

10(a) Opithosoma (abdomen) distinctly segmented; body flattened dorso-ventrally; *large (up to 5 mm)*; *pedipalps chelate (pincer-like).* _____ **PSEUDOSCORPIONIDA (Pseudoscorpions)**

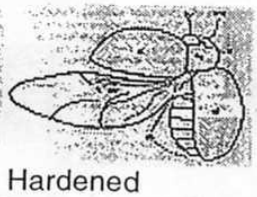


(b) Opisthosoma unsegmented; small animals. _____ **ACARI (Mites)**

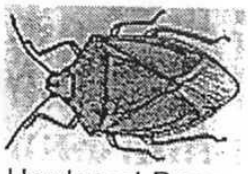
11(a) *Wings* well developed. _____ 12
(b) *Wings absent.* _____ 17

12(a) Wings membranous. _____ 15
(b) Front wings hardened or leathery, at least at base. _____ 13


Membranous

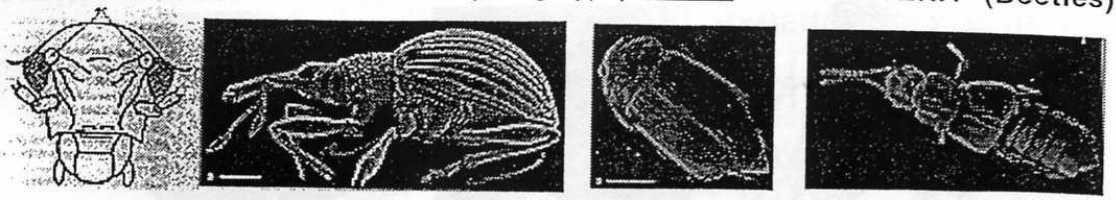

Hardened


Hardened


Hardened Base

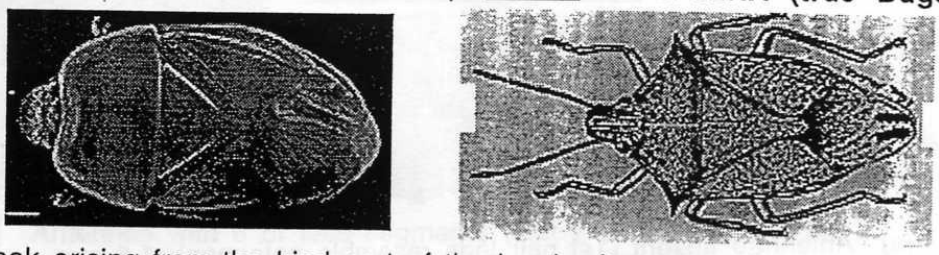
6

13(a) Mouthparts mandibulate (biting type)._____ **COLEOPTERA (Beetles)**

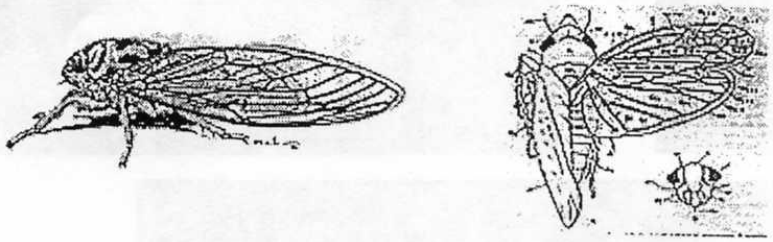


(b) Mouthparts haustellate (sucking type). _____ 14

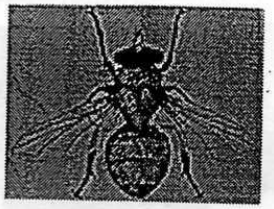
14(a) Beak arising from the front of the head; front wings usually thickened at the base & membranous at the tip. _____ **HEMIPTERA (true Bugs)**



(b) Beak arising from the hind part of the head, often appearing to originate at the base of the front legs; front wings uniformly textured.
_____ **HOMOPTERA (Aphids, Psyllids etc.)**

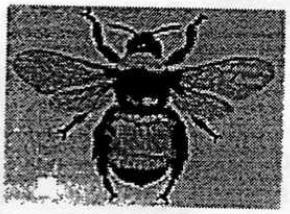


15(a) 1 pair wings; second set wings reduced to halteres. _ **DIPTERA (Flies)**

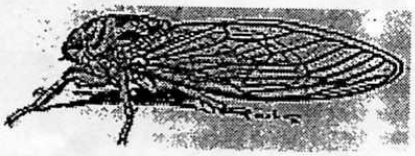




(b) 2 pair wings. _____ 16

16(a) Body petiolate; mouthparts mandibulate. _____
_____ **HYMENOPTERA (Bees/Wasps etc.)**

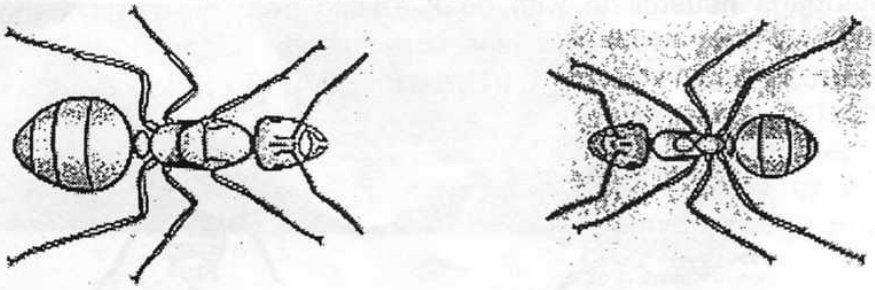

(b) Body not petiolate; mouthparts haustellate. _____ **HOMOPTERA**




17(a) Antennae absent (1st pair legs resemble antennae); 1.5 mm or less.
_____ **PROTURA (Proturans)**
[see "8a" for figures/photos]

(b) Antennae definately present. _____ 18

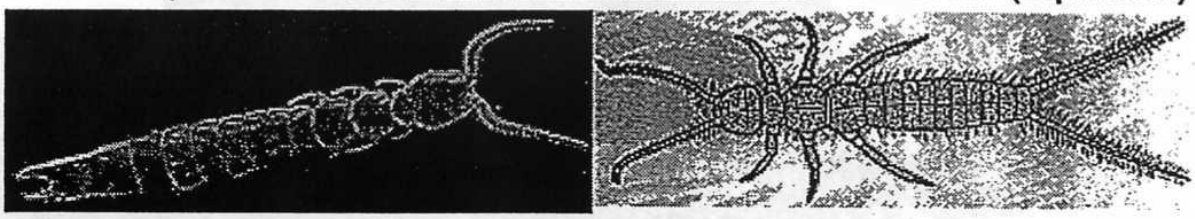
18(a) Abdomen constricted at base (petiolate); antennae usually elbowed; hard bodied. _____ **HYMENOPTERA (Ants etc.)**



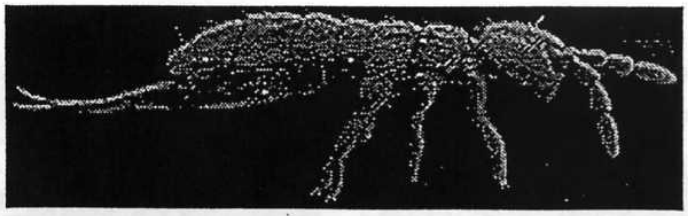
(b) Not as above. _____ 19

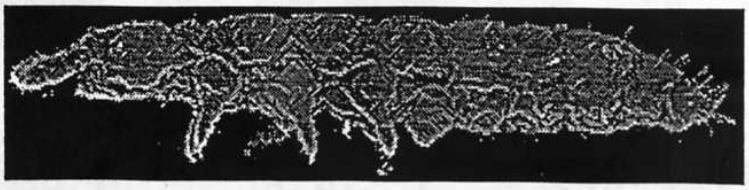
19(a) Mouthparts withdrawn into the head & not appearent. _____ 20
(b) Mouthparts usually distinct. _____ 21

20(a) Antennae long & multi-segmented; abdomen with at least 9 segments & with style-like appendages on ventral side of some segments; well developed cerci. _____ **DIPLURA (Diplurans)**



(b) Antennae with 6 or less segments (last 2 segments may be annulate); abdomen with 6 or less segments; furca often obvious. _____ **COLLEMBOLA (Springtails)**





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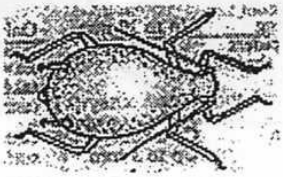
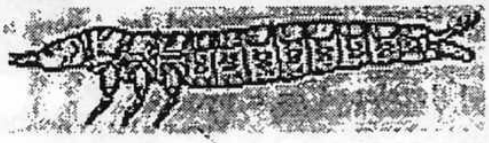
21(a) Mouthparts haustellate with beak arising from the rear of the head; antennae usually with more than 5 segments; abdomen often with a pair of cornicles. _____ **HOMOPTERA (Aphids, Scale Insects etc.)**



(b) Mouthparts mandibulate; antennae usually short if present. _____ **INSECTA LARVAE**

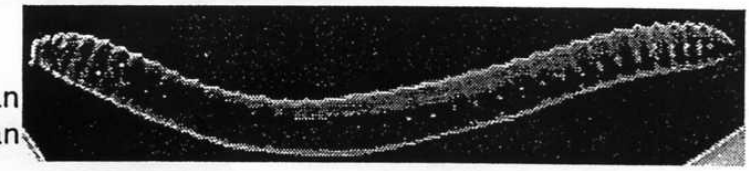


10

OTHER ORGANISMS FREQUENTLY FOUND IN B.C. FOREST SOILS.

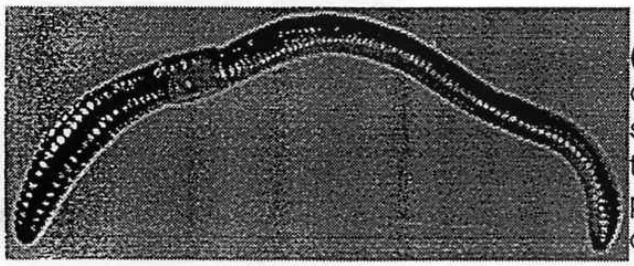
NEMATODA

(Roundworms) - typically very small (<1mm); straight or curled in shape; often transparent or white in color; found in large numbers.



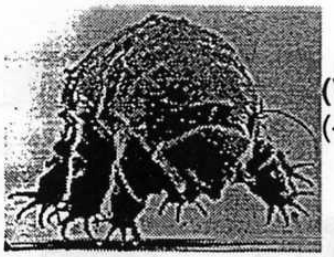
ENCHYTRAEIDS

(Pot worms) - larger than nematodes, but smaller than earthworms (rarely more than 25 mm); segmented; usually white or pinkish; usually elongated or cylindrical; the chaetae (hairs/bristles) occur in bundles.



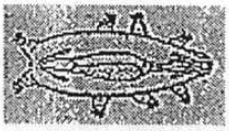
ANNELIDA: LUMBRICIDAE

(Earthworms) - large (17-300 mm); cylindrical; tapering at both ends; segmented; a saddle-shaped thickening called a clitellum is present in all mature earthworms; color varies but often brownish.



TARDIGRADA

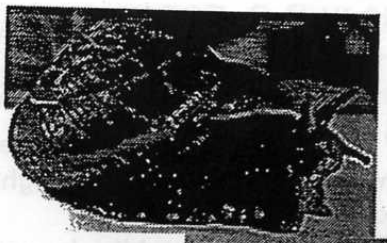
(Water bearers) - very small (<1mm);



ROTIFERA

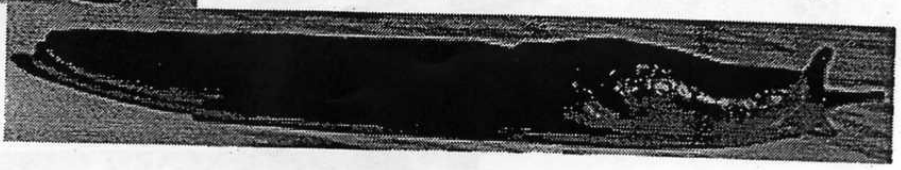
(Rotifers)

very small (<1mm);



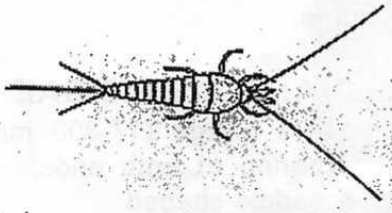
MOLLUSCA: GASTRODODA

(Slugs & Snails) - muscular "foot" for locomotion - no legs; no obvious external segmentation; most with 2 pair retractable tentacles at anterior; some with a shell.

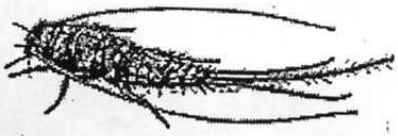


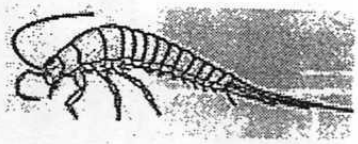
Thysanura

(Bristletails)



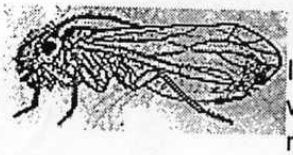
Archaeognatha: Insecta. 3 pair legs; 10-15 mm long; no wings; body usually elongate & somewhat flattened; long antennae; *a long central terminal filum (tail) that projects further than paired cerci (shorter tails)*; only superficially adapted to soil.



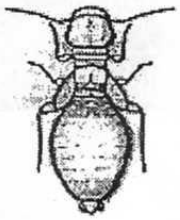


Psocoptera

(Bark-lice)



Insects; 3 legs; wings present or absent; winged forms have 2 pair of wings that at rest are usually held like a roof- front wings slightly larger than rear wings; antennae generally fairly long; no circi; mandibulate mouthparts; live on or under bark, in foliage, under stones or in dead leaves.



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Isoptera (Termites)

Insects. 3 legs; wings present or absent - when present, there are 4 membranous wings approx. same size and shape (ants front wing s are larger than their rear wing) that at rest are held over the body and extend beyond the end of the abdomen; mouthparts mandibular; bodies soft & usually light colored (not hard and dark like ants); abdomen broadly joined to thorax (not constricted as ants); antenae are usually moniliform or filiform (ants are elbowed).

Brachipoda - CRUSTACEA.

Copepoda - CRUSTACEA: Harpacticidae. Cephalothorax & abdomen not separated; first antennae short, at most 8-membered; fifth pair legs rudementary, plate-like & developed differently in the male & female; with wet dead leaves; predators.

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Acknowledgements

Faunal keys are from the following publication

STUDENT KEY TO B.C. FOREST SOIL ANIMALS

1994

by
Marilyn R. Clayton

prepared for

Canadian Forest Service
Pacific Foresty Centre
506 West Burnside Road
Victoria, B.C.
V8Z 1M5

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