Bioprospecting — Filter Paper Assay Method

Overview: Students collect samples that they predict will contain communities of cellulose-degrading microbes and test for the ability of microorganisms in their samples to break down pure cellulose (filter paper). In the process, groups collect evidence to test predictions about which environmental microbial samples will be the most effective for degrading cellulose. By comparing results across groups, students can begin to uncover patterns and develop explanations about the types of environments that support cellulose-degrading microbes. This lab method is nearly identical to that used by GLBRC researchers, and student results could help scientists discover new enzymes for efficient cellulosic biofuel production.

Levels
High School-Undergraduate

Subjects
Science, Biotechnology, Environmental Science, Agriculture

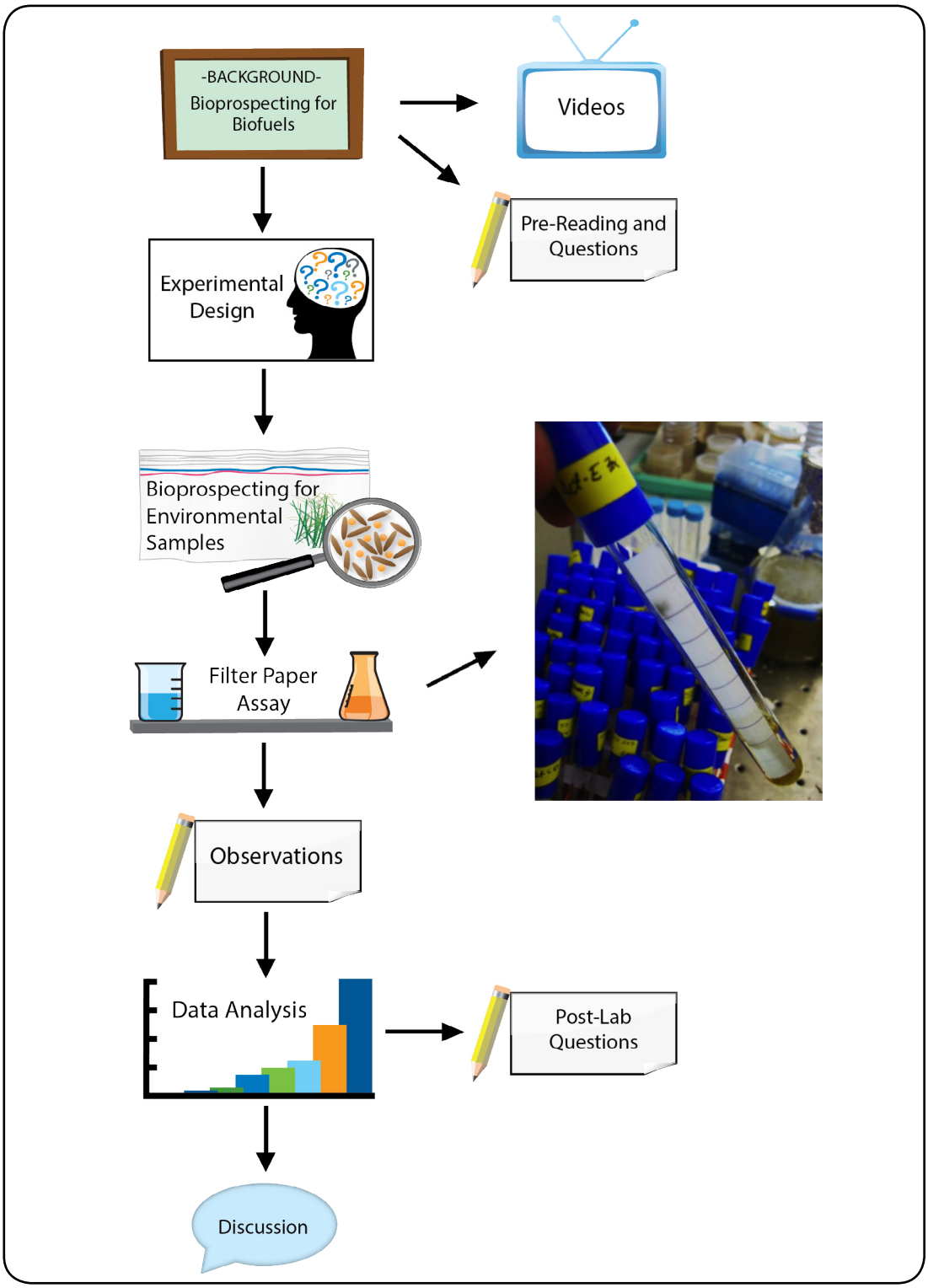
Objectives
- Explain the relationship between plant cellulose and cellulase enzymes
- Describe the general process for converting cellulosic biomass into ethanol
- Explain the function of cellulase enzymes for converting cellulose into biofuel
- Describe the role of cellulose-degrading microbes in biofuel production
- Predict, test, analyze, and explain the effectiveness of different microbial populations for degrading cellulose
- Describe and explain general patterns in evironmental characteristics that harbor cellulose-degrading microorganisms

Materials
Bioprospecting package

Activity Time
Three to five 50-minute class periods over 10-14 days

Standards
Next Generation Science Standards (2013)
- Scientific and Engineering Practices: planning and carrying out investigations; analyzing and interpreting data; constructing explanations and designing solutions
- Disciplinary Core Ideas: ecosystems; engineering design
- Crosscutting Concepts: patterns; cause and effect; energy and matter
- Performance Expectations: See page 3 for details

NGSS Lead States. 2013. Next Generation Science Standards: For States by States. Washington DC: The National Academies Press



For Teachers: Bioprospecting for Cellulose-Degrading Microbes — Filter Paper Assay Method

Overview:

Students collect samples that they predict will contain communities of cellulose-degrading microbes and test for the ability of microorganisms in their samples to break down pure cellulose (filter paper). In the process, groups collect evidence to test predictions about which environmental microbial samples will be the most effective for degrading cellulose. By comparing results across groups, students can begin to uncover patterns and develop explanations about the types of environments that support cellulose-degrading microbes. This lab method is nearly identical to that used by GLBRC researchers, and student results could help scientists discover new enzymes for efficient cellulosic biofuel production.

This lesson is designed to span three to five 50-minute class periods over 10 to 14 calendar days depending upon student familiarity with cellulosic biofuels. The majority of class time is required to provide background information, plan investigations and interpret results. Samples should incubate for 7-14 days while students record periodic observations. This activity provides for flexibility depending upon time constraints, student prior knowledge, equipment and funding.

Learning Outcomes: Students will…

- Explain the relationship between plant cellulose and cellulase enzymes
- Describe the general process for converting cellulosic biomass into ethanol
- Explain the function of cellulase enzymes for converting cellulose into biofuel
- Describe the role of cellulose-degrading microbes in biofuel production
- Predict, test, analyze, and explain the effectiveness of different microbial populations for degrading cellulose
- Describe and explain general patterns in environmental characteristics that harbor cellulose-degrading microorganisms

This lesson assumes some prior knowledge of types of carbohydrates, basic enzyme function, the process of decomposition, the role of microorganisms in ecosystems, and matter and energy transformations.

Standards

Next Generation Science Standards (2013)

Performance Expectations

Middle School:

- **MS-LS2-1.** Analyze and interpret data to provide evidence for the effects of resource availability on organisms and populations of organisms in an ecosystem.
- **MS-LS2-3.** Develop a model to describe the cycling of matter and flow of energy among living and nonliving parts of an ecosystem.

High School:

- **HS-LS2-7.** Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.
- **HS-ETS1-2.** Design a solution to a complex real-world problem by breaking it down into smaller, more manageable problems that can be solved through engineering.

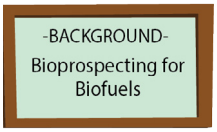
Scientific and Engineering Practices	Disciplinary Core Ideas	Crosscutting Concepts
Planning and carrying out investigations Analyzing and interpreting data Constructing explanations and designing solutions	**LS2**: Ecosystems: Interactions, energy, and dynamics **ESS1**: Engineering design	Patterns Cause and effect: Mechanism and explanation Energy and matter: Flows, cycles and conservation

See Appendix for alignment with other standards.

Master Materials List

Item	Suggested Quantity
Test tubes w/ stoppers, parafilm, or Falcon 50mL tubes	4/group
Miracle Gro 20:20:20 Fertilizer	20g/class
Whatman Filter Paper (cellulose)[1]	4 1x10cm strips/group
Test tube rack	1/class
Test tube shaker[2]	1/class
Graduated cylinders	1/group
Ziploc bags (snack sized)	4/group
Markers	1/group
Labeling tape	1/class

1. High-grade cellulose filter aper circles can be ordered from Ward Scientific for approximately $12. *https://www.wardsci.com/store/catalog/product.jsp?catalog_number=153701&sk=1*
2. Orbital shaker for use on stir plate for approximately $300. *https://www.wardsci.com/store/catalog/product.jsp?catalog_number=158065*

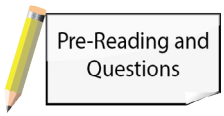


Sequence:

Part 1. Background Information: Framing the Problem (Pre-Reading Assignment, Videos) (1-2 50-minute periods)

Bioprospecting and Biofuels for Beginners: A TED-Ed flip classroom lesson was created with Craig Kohn to accompany this lab activity. Students can watch the online video to introduce the important challenge of making cellulosic ethanol and the larger context for this lab activity. View the video and lesson here: *http://ed.ted.com/lessons/biofuels-and-bioprospecting-for-beginners-craig-a-kohn*

OPTIONAL: Have students complete online flip lesson with questions and discuss student responses to the online questions during class (*http://ed.ted.com/lessons/biofuels-and-bioprospecting-for-beginners-craig-a-kohn#review*).



Pre-Reading and Pre-Lab Questions: Students can complete the reading and accompanying questions as homework or in their lab groups. Have students discuss answers in their lab groups and then review responses in a class discussion. The accompanying PowerPoint slides can be used to review the structure of cellulose, the function of cellulase enzymes and the process of making cellulosic ethanol. In particular, question #6 is valuable to discuss in lab groups and as a class.

As students consider environmental and microbial characteristics that would be associated with effective cellulose breakdown, they will begin planning where to search for environmental samples and develop reasoned predictions about which samples will be the most effective.

Bioprospecting and Biofuels - Connecting Classrooms with GLBRC Research: The video interview with Gina Lewin, microbiology PhD student at UW-Madison, provides a context for how this lab was developed and how the techniques used by the students are very similar to those used by GLBRC scientists. Gina worked with Craig Kohn to develop this activity as part of the GLBRC Research Experience for Teachers program. The leaf-cutter ant example provides a useful case-study for how cellulose-degrading microorganisms, such as fungi, play an important role in ecosystems but also produce cellulase enzymes which could be valuable for biofuel production. View the video here: *https://vimeo.com/72999628*

Bioprospecting Lab Introduction with Craig Kohn: This video can be used as a teacher reference or shown to students to provide a description of the lab protocol. The video is divided into sections that describe each portion of the lab sequence so you can play specific segments to your class before starting a lap step. View the video online: *https://vimeo.com/73143224*

Part 2. Planning and Collecting Samples (1-2 50 minute class periods) (pages 1-4)

Set-up:

The process of reviewing the lab protocol and setting up test tubes helps students learn how this method will allow for detection of cellulase activity. As you describe the lab set-up, discuss the purpose of the filter paper and liquid media. Have students discuss what evidence they might see that would indicate the presence or absence of high numbers of cellulose-degrading microbes in their samples.

Demonstrate the proper test tube set-up as described in the student lab and shown in the supplementary video. Emphasize the importance of sterile procedure and avoiding contamination of the test tubes and filter paper. We want to be sure that any signs of cellulase activity are resulting from microbes in the environmental sample and not microbial contamination. Even so, it is unlikely that small amounts of contamination coming from students hands and the classroom will contain sufficient numbers of cellulose-degrading microbes to produce positive results.

It is important that the filter paper strips lie flat against the side of the test tube so that they don't fall into the fertilizer media making difficult to detect the degree of filter paper degradation. If necessary, a strip of tape can be used to secure the filter to the side of the tubes.

If possible, have students set up four test tubes so that they will have a replicate of each sample. To save materials and space, groups can set up only one tube of each sample, but as a result, students will not see the variation in results between tubes with the same environmental sample. It is common in this lab to see variable results (positive and negative) for replicates of the same environmental sample.

Control test tubes: Set up several test tubes to serve as controls. Either do not inoculate these tubes or, preferably, add an environmental sample that has been autoclaved to kill all microbes. The controls will serve as a point of comparison so students can gauge the degree to which their filter paper degraded.

Media Solution: The media solution is necessary to provide non-energy related nutrients for microbial cell growth. Either have students mix quantities of the solution or prepare a batch for them. The fertilizer recipe is chemically similar to the media used by GLBRC scientists.

Miracle Grow Media Recipe: (developed by Craig Kohn, 2011 – GLBRC) – add 20 g of 20:20:20 Miracle Grow to 1 liter of tap water (pure water is not recommended because it has fewer minerals that can aid microbial growth). This solution will settle if not agitated, so be sure to swirl and re-suspend before adding samples. 20:20:20 Miracle Grow contains 20% Nitrogen, 20% Phosphorus, and 20% Potassium. Other formulations of Miracle Grow will have different concentrations of these nutrients and are therefore not recommended. Other brand names of 20:20:20 fertilizer should work equally well assuming that they have no organic ingredients. If a fertilizer does have organic ingredients, it cannot be used because it will provide a source of energy outside of the cellulose, preventing the test from providing accurate results.

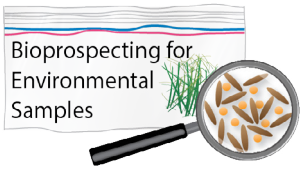
Planning:

Remind students of the goal of the investigation: to find samples that have high concentrations of microbes that can rapidly degrade cellulose. To begin the planning process, students can discuss in small groups some of the environmental characteristics that would contribute to samples being effective for degrading cellulose (question #6 in pre-reading activity). Follow-up small group discussion with class discussion in which students share and explain proposed characteristics. These characteristics can be summarized on the board.

Below are some of the characteristics groups might consider. There is no single set of correct answers to this prompt. The goal is for students to reason from basic principles about conditions for active decomposition in the environment.

Evironmental Characteristic	Reasoning/Evidence
High concentrations of cellulosic biomass	Serves as food (energy) source for cellulose-degrading microbes
Evidence of decomposing cellulosic biomass	Cellulose-degrading microbes are actively consuming this biomass
Moist conditions	Water is required for organism growth and development
Warm conditions	Microbial growth and reproduction increases in warm conditions

Considering the shared list of environmental characteristics, students should brainstorm with their groups a list of potential locations to bioprospect.



Bioprospecting for Samples:

Refer to the accompanying video for additional instructions on how to collect samples. Students can collect their four samples in groups during class or individually for homework. Have students consider how to care for their collected samples so that the microbes remain active. For instance, if a sample were collected in a cool, moist location and then left in a hot car over the weekend, many of the microorganisms might not survive to the inoculation stage. Samples can be stored in the fridge if inoculation is delayed.

Choosing a Sample and Making Predictions:

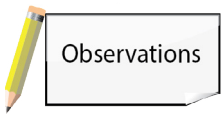
Lab groups should decide which of the four samples collected will show the most cellulase activity. Have groups discuss and develop explanations for their choice and then share their reasoning with the class. Next, students can complete the planning and comprehension questions on pages 3-4. These questions can serve as a formative assessment to determine how well they understand the experimental design. Students should make reasoned predictions that they can revisit and reevaluate as they collect and interpret data.

Part 3: Sample Inoculation and Observations (one 50-mintute class period and brief observations over 7-14 days) (pages 7-10)

Use the accompanying video and demonstrations to illustrate proper inoculation technique and remind students to follow sterile procedures to avoid contamination. Also remind students to keep the tubes upright. Try to avoid hitting the filter paper with the sample. In this way, any filter paper degradation should start in the media solution and work upward.

Before adding tubes to the shaker, have students record initial observations. If possible, take photos with observations to document any changes. Review the potential indicators of cellulase activity in the samples and the causes for those changes.

Observations: This lab can be ongoing for 1-2 weeks while doing other activities. Students should make brief observations every 1-2 days and can periodically share results through the course of the experiment. For samples with active cellulase-degrading microbes, expect to see evidence of filter paper tearing within 5-10 days.



One method to quantify the amount of cellulase activity is in a sample is to count the number days until a complete tear is observed in the filter paper. Students should record the date that partial and complete tears in the filter paper are seen so that the degree of cellulase activity can be compared between samples.

Test Tube Shaker: A shaker 1) increases microbial contact with the filter paper and 2) aerates the media. This increases the rate of filter paper decomposition. It is possible to use this assay without a shaker but degradation could take a month or more. GLBRC scientists put the test tubes on an angle in the shaker which increases the liquid-air surface area, thus improving aeration. This can be done to speed up the degradation process, but is not necessary.

Aerobic vs. Anaerobic Conditions: Note that if the test tubes are completely sealed, anaerobic conditions will exist. Conditions in a tube will select for different subsets of the microbes in the samples based on whether they are aerobic or anaerobic. In general, cellulose decomposition will occur more rapidly in aerobic conditions, but it also occurs in anaerobic conditions (i.e. cow's rumen). If test tubes are left partially open, students should monitor media solution levels because some of the water can evaporate.

Part 4: Data analysis, Discussion and Conclusions (pages 8-10)

At the end of the observation period, groups can summarize their observations and conclusions by completing the tables and questions in the Lab Analysis and Comprehension section of the student handout. Students should be able identify which samples showed evidence of cellulose-degrading microbe activity and what caused any observed disintegration in filter paper, i.e. microbes in the sample were able to produce cellulase enzymes, break down the cellulose into glucose and consume the glucose for energy. Likewise, they should be able to explain what causes other test tubes to show no evidence of cellulase activity.

Groups should review their initial predictions and determine how evidence collected in the lab either supports their predictions, refutes them, or is inconclusive. Students should draw on prior knowledge, background readings and supplementary research to propose reasonable explanations for inconsistencies between what was predicted and what was observed. There are numerous factors that affect results in this lab (i.e. microbe pH and temperature tolerance, unpredictable competitive interactions and population dynamics in microbe communities, community variation between replicates of the same environmental sample). It is less important for students to come up the correct explanation for observations than to ask questions, seek answers, reason from evidence and draw on plausible biological principles.

To revisit the discussion about which environmental characteristics should support cellulose-degrading microbes, have groups share results. This can be summarized on a table on the board or in a shared online document. One method would be to have the class report how their samples fall into the three categories:

Definite Growth	Possible Growth	No Growth

For more quantitative comparison to determine which samples had the greatest cellulase activity, a table might be organized as shown below. The samples that showed a complete tear in the filter paper the soonest would be the most active.

Group Name	Sample Description	Definite, Possible, or No Growth	# Days to Complete Tear in Paper	Length of Paper Decomposed

Discussion

In groups, have students consider what the samples in each category (definite growth, possible growth, no growth) have in common. In particular, they should consider differences in environmental characteristics that could account for the patterns seen in which samples show evidence of cellulase activity. Have groups share their observed patterns and proposed explanations with the class. Discuss how understanding these patterns could help researchers discover new cellulase enzymes that could be used in biofuel production.

Note on interpreting results: When comparing cellulase activity between samples, the best overal indicator is number of days until a complete tear is observed. The fewer the days, the more activity. The position or length of the tear does not necessarily indicate more or less cellulase activity. Available oxygen and nutrients vary along the length of the filter paper which can cause certain microbes to more active and create a tear at different positions.

It is important to remember that you are observing the effects of a community of microbes growing in the test tube. There are numerous species of microbes interacting the sample. More effective filter paper degradation could be caused by either by a larger, more diverse community of microbes that can produce cellulase enzymes or it could be caused by relatively smaller number of microbes that are highly effective at degrading cellulose.

Expected results: Based on conducted lab and classroom trials, filter paper decomposition is frequently observed from soil samples, decomposing wood, compost, and ruminant manure. In very active samples, filter paper decomposition can occur in less than a week. However, as mentioned previously, there can be a great deal of variation in observed cellulase activity between similar samples.

Share your results with GLBRC: GLBRC researchers would be interested in learning about samples in which you observed cellulase activity, especially those environmental samples that can degrade filter paper in less than seven days. In addition, by looking at results from samples collected across a geographic range, scientists might be able uncover broader patterns about how cellulase activity changes with soils or latitude.

Share your results with this online form: *https://glbrc.wufoo.com/forms/share-your-bioprospecting-results-with-us/*

Extensions and Options:

This lab protocol can be simplified or expanded to include more or less complexity depending upon the goals, classroom environment and audience. Ideas to simplify include:

- Conduct the lab as a demonstration and have students predict and explain results.
- If a shaker is not available, it is possible to set up samples in tubes and let them sit for a month or more and make observations every week. This experiment could be going on in the background of a class for a semester.
- Provide a variety of environmental samples for students to choose from rather than allowing them to collect their own.

Possible extensions:

- Isolate microbes from active samples using the GLBRC Isolation Method protocol: *http://www.glbrc.org/education/educationalmaterials/bioprospecting*
- In samples that are proven to be effective at cellulose breakdown, conduct trials to evaluate the effect of variables such as oxygen availability, temperature, and inoculation sample size on the results.
- Organize as cooperative class or smaller group investigation of a research question. In this scenario, students could develop and evaluate a scientific claim through the course of the investigation. Here are some example questions:
  - How does cellulase activity change with soil type?
  - Which fungus species show more cellulase activity?
  - Do samples from decomposing wood show more cellulase activity than herbaceous plants?

Appendix

Video Resources:

Bioprospecting and Biofuels for Beginners. This short video, developed by H.S. Teacher Craig Kohn and TED-Ed animators, provides an excellent introduction to the concept of bioprospecting and potential use for developing sustainable biofuels: *http://ed.ted.com/lessons/biofuels-and-bioprospecting-for-beginners-craig-a-kohn*

Bioprospecting and Biofuels - Connecting Classrooms with GLBRC Research. This interview with microbiologist and biofuels scientist, Gina Lewin, describes how this lab activity connects to the bioprospecting investigations of leaf-cutter ant communities that could lead to discoveries of new enzymes for more efficient biofuels: *https://vimeo.com/72999628*

Introduction to Bioprospecting Lab and Protocol. Craig Kohn, the teacher who worked with GLBRC to create this activity, describes and demonstrates the steps of the lab protocol. This video can used as a teacher reference or can be shown to students in sections to introduce the steps of the lab protocol: *https://vimeo.com/73143224*

Bioenergy in the High School Classroom. Bioprospecting Lab. In this narrated slideshow, Craig Kohn describes the process of developing the lab and his experiences using it with his students: *http://vimeo.com/43991600*

Text/Online Resources:

Why is it so difficult to make cellulosic ethanol? This short reading introduces the challenges and opportunities associated with producing ethanol from cellulosic biomass. The reading includes details about the chemical composition of plant cell walls: *http://www.glbrc.org/sites/default/files/Cellulosic_Ethanol.pdf*

Researchers Unearth Bioenergy Potential in Leaf-cutter Ant Communities. This GLBRC research news article describes how GLBRC scientists are identifying new enzymes produced by fungi in leaf-cutter ant colonies that might used to make cellulosic biofuels: *http://www.glbrc.org/?q=node/2042*

The Ant Man: UW scientist Cameron Currie looks to the insect world for insights on how to save the planet. This entertaining news article describes GLBRC scientists' work in unraveling the complex symbiotic relationships in fungus-farming ant communities that could offer clues for developing cellulosic biofuels: *http://www.isthmus.com/isthmus/article.php?article=28844*

I-MOLD Animated Lessons on Leaf Decomposition. The animations allow you to visualize the matter transformations associated with decomposition at the cellular and molecular scale: *http://imold.utoledo.edu/lessons.html*

Standards:

WI MODEL ACADEMIC STANDARDS (SCIENCE GRADE 12):

B.12.4 Show how basic research and applied research contribute to new discoveries, inventions, and applications
C.12.3 Evaluate the data collected during an investigation, critique the data-collection procedures and results, and suggest ways to make any needed improvements
C.12.5 Use the explanations and models found in the earth, space, life, environmental, and physical sciences to develop likely explanations for the results of their investigations
F.12.7 Investigate how organisms both cooperate and compete in ecosystems
F.12.10 Understand the impact of energy on organisms in living systems

AGRICULTURE, FOOD AND NATURAL RESOURCE STANDARDS:

BS.02.05.06.a. Explain reasons for detecting microbes and identify sources of microbes
BS.03.03.08.c. Creation of biofuels from biomass
BS.03.03.09.c. Biotechnology processes & molecule synthesis
ESS.01.01.01.a. Explain the importance of unbiased sampling and collect samples
ESS.01.01.01.c. Analyze/interpret results of samples
EES.02.05.06.c. Design and perform an assay to detect a target microorganism in food, water or the environment

AAAS PROJECT 2061 (1993):

1B - The Nature of Science: Scientific Inquiry
3C - The Nature of Technology: The Issues in Technology
5D - The Living Environment: Interdependence of Life
5E - The Living Environment: Flow of Matter and Energy
12C - Habits of Mind: Manipulation and Observation
12D - Habits of Mind: Communication Skills

See page 3 for Next Generation Science Standards (2013) alignment.

Activity was developed with Craig Kohn, Waterford Union High School, Waterford, WI while working with Gina Lewin in Dr. Cameron Currie's Lab at the University of Wisconsin-Madison.
Funding and additional support provided by the Great Lakes Bioenergy Research Center

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WWW.GLBRC.ORG/EDUCATION
EDUCATION@GLBRC.WISC.EDU

U.S. DEPARTMENT OF ENERGY