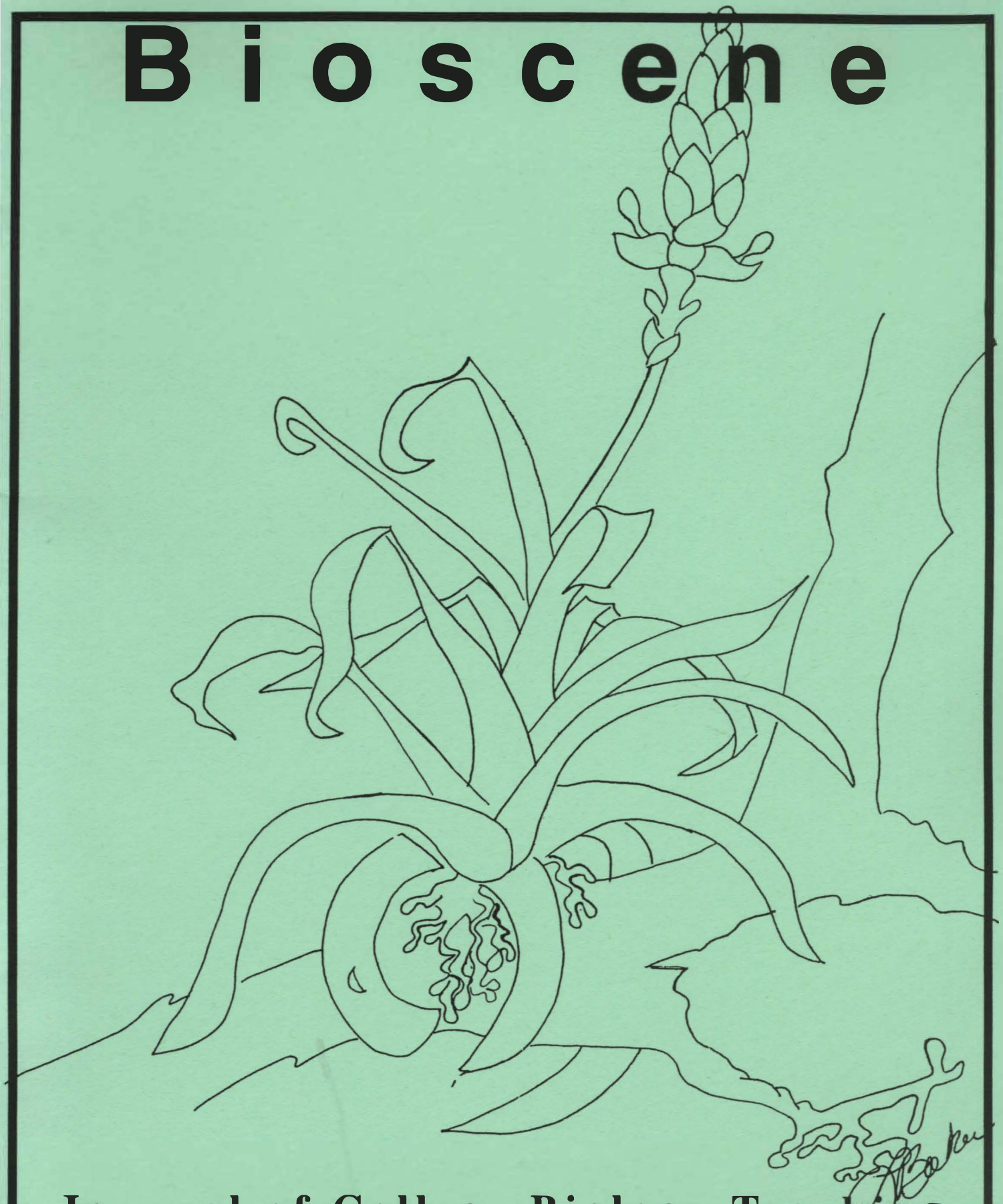
Bioscene



Journal of College Biology Teaching
Volume 21(3) • November 1995

Bioscene
Journal of College Biology Teaching
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Table of Contents

Articles:

News & Views:

Cover Illustration: Bromeliads drawn by Laurie Baker, Senior Biology student at Park College, Parkville, MO 64152-3795. Contributed by Dorothy May.

Bioscene: Journal of College Biology Teaching

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Suggestions for Manucripts

Announcements	Sources of Funding	Biology Policy Issues	Reviews of
Letters to the Editor	Sociology of Biology	Minority Education	Software/Hardware
Book Reviews	Courses that Work	Women in Biology	Philosophy of Biology
Laboratories that Work	Professional School	Computer Advice	History of Biology

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ECOLOGICAL MODELING AS A FORUM FOR STUDENT DECISION-MAKING

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Wise decision-making may be the most important capability our students possess upon completing a degree in biology. Risk assessment, cost-benefit analysis, resource valuation and problem solving in general require an ability to process and filter information (Ost 1995). Further, the ability to connect seemingly disparate bits of information often leads to the truly interesting and valuable discoveries in science (Bicak and Bicak 1990). This ability is generally not innate (Bohn 1980), but rather must be learned. We as teachers in science have long contended that students learn best by doing. Scientific inquiry is more an active experiential *process* and less a passive observation of the *products* of science.

The modeling process lends itself well to interactive student involvement. Learning is most comprehensive when students actually make and evaluate models rather than simply hear about them from an instructor (Jungck and Calley 1985; Horton and Bicak 1987). Computer models help us to understand the dynamics of real systems and importantly, to identify what we don't know as well as what is known (Hannon and Ruth 1994). Modeling proficiency requires:

1) determining appropriate initial conditions (with which numbers do we start?);
2) documentation (what do the numbers mean?);
3) sensitivity analysis (what factors influence the numbers most?); and
4) validation (are the output numbers from the model meaningful?)

Ost (1987) recognizes four classes of models: representative, analog, logical and theoretical. While biologists use all types, most emphasis is placed on theoretical models which are mathematical and thus, quantitative and predictive in nature.

Success in modeling required the modeler to slide along the scale of Bloom's taxonomy (1956). Cognitive levels of expected student competency are:

1) knowledge:	questions which ask what, when, where, who
2) comprehension:	compare, contrast, describe
3) application:	solve, choose, classify
4) analysis:	analyze, provide evidence, identify reasons
5) synthesis:	predict, develop, design
6) evaluation:	assess, judge, appraise

Collete and Chiapetta (1989) report that teachers who understand the taxonomy promote student mastery of a variety of cognitive processes. To do less, would seem to be a disservice to the students.

Our suggestion is not to abandon the textbook, but rather to integrate decision-making exercises into courses in a way that complements information with its skilled use. Our report concerns the use of simulation modeling in ecology as a means to achieve this goal.

Systems in Science

A system is a grouping of interactive and interdependent parts. This implies that exchange takes place among the parts of a system. Exchange may be identified as energy, matter or information. Regardless of the currency (e.g. joules, grams of carbon, hormonal signals) used to characterize a system, the definition works well for both physical and biotic scenarios. J. W. Forrester (1968), an electrical engineer, would add that the parts of a system operate together for a common purpose. While the concept of purpose is arguable in biological systems, there is no question that ecologists have borrowed heavily from both engineering and management science in the maturation of systems ecology. The broad definition of a system allows inclusion of an automobile engine, a set of pulleys and levers and an ecosystem in the same arena. What separates biological from physical

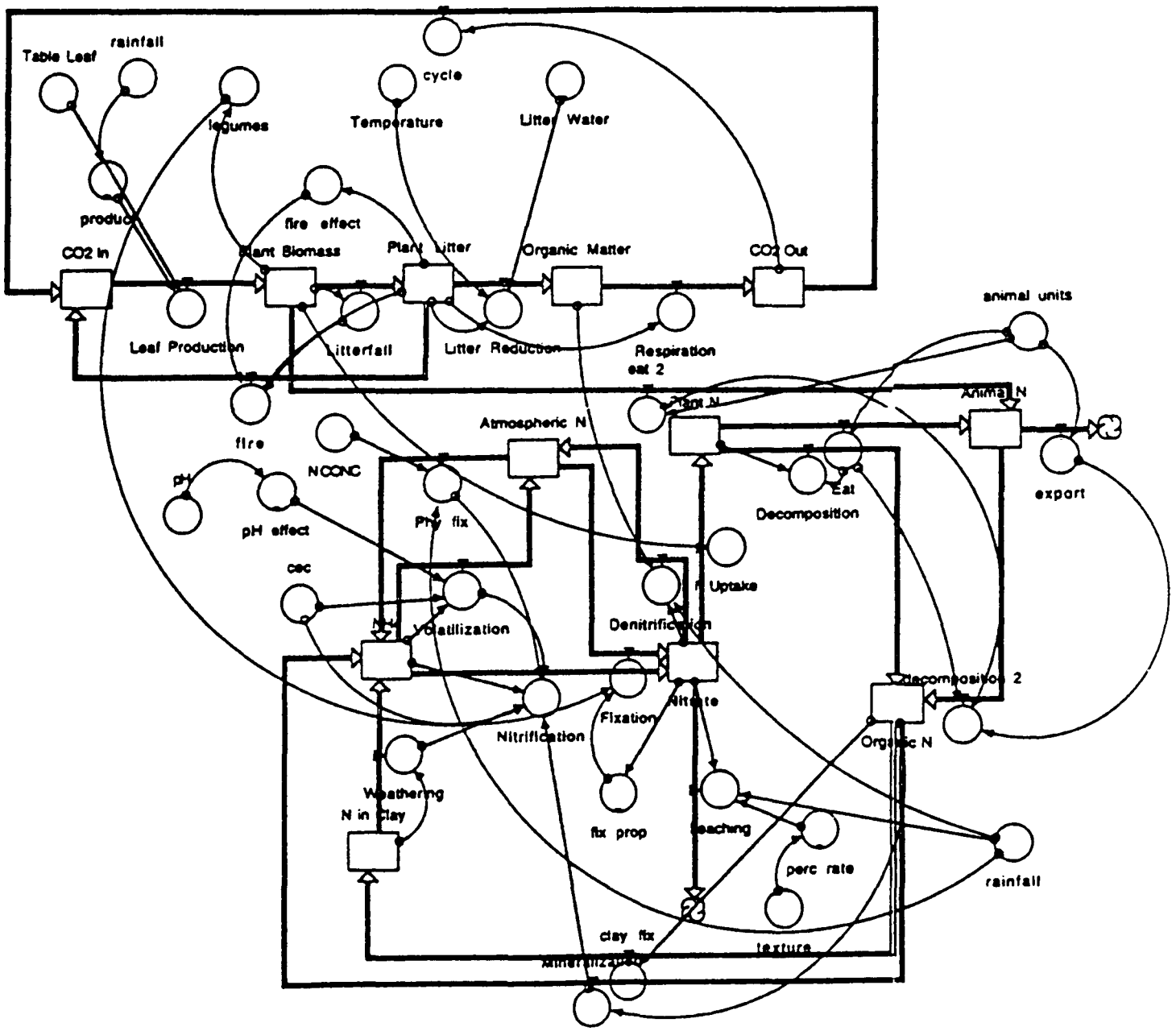

Figure 1. Working model of the Willa Cather Prairie. Boxes are state variables or amounts. Open arrows are flows or rates. Circles on flows are regulators or controllers of flows. Solid arrows are information lines indicating influence of driving variables or detached circles on flows. The model includes five state variables and seventeen driving variables.

systems is the living element: notably plants and animals. The living element, however, is a very real wild card. Predicting change in ecosystems is difficult enough. However, if we add the human element as in political, economic and social systems, then the reliability to predict change becomes extremely challenging. Still, the benefit in terms of improved understanding and wise stewardship warrants the cost of investment in biological systems research.

Grassland Model: Willa Cather Prairie

Our goal was to design, construct and implement a computer simulation model with reliable forecasting properties for key producer, consumer and decomposer interrelationships and dynamics in the Willa Cather Prairie. This prairie preserve is representative of the southern mixed grass prairie, the potential natural vegetation that extends from south central Nebraska through central Kansas and Oklahoma and into north Texas (Nicholson and Marcotte 1979). Much of the natural grassland has been displaced by agriculture; however, the Willa Cather Prairie is a subatantial (247 ha) exclosure with minimal, including human, disturbance in the last eighteen years. Vegetation includes perennial native warm season grasses such as *Andropogon gerardi* (Big bluestem), *A. scoparius* (Little bluestem), *Bouteloua gracilis* (Blue grama) and *B. curtipendula* (Sideoats grama). Perennial native cool season grasses include *Agropyron smithii* (Western wheatgrass) and *Sporobolus asper* (Tall dropseed). *Poa pratensis* (Kentucky bluegrass) and *Bromus inermis* (Smooth brome) are two perennial introduced cool season grasses that contribute significantly to the prairie productivity. In all, 236 species of vascular plants were identified in the first comprehensive survey of the prairie (Nagel 1977). The landscape is characterized as rolling loess hills. Fire has historically been an important influence on plant species composition and productivity (Nagel 1994). There is no history of cultivation on the Willa Cather Prairie. It offers an ideal case study for simulation of mixed grass prairie dynamics.

Several specific objectives were stated in the modeling project:

1) designate abiotic and biotic submodel components (e.g,. precipitation, temperature, primary productivity, nitrogen processing, etc.);
2) document and validate the model;
3) assess the sensitivity of selected components of the model; and
4) integrate the model into an upper division ecology course.

This last objective required the model to be "transportable" from our desks to those of the students.

We designed a working model of the Willa Cather Prairie (Figure 1) with five state variables (boxes) that simulate carbon flow and seven that simulate nitrogen flow. Seventeen driving variables (circles) were designated as regulators or controllers on flows. State variable typically constitute dependent variables while driving variables are independent. The model was fashioned using STELLA®; which is an acronym for "Systems Thinking, Experiential Learning Laboratory, with Animation." This software allowed us to construct biological models with legitimate descriptions *and* prescription powers. While other software packages are available for modeling, STELLA® is nicely designed to allow an intuitive approach to model construction. Further, the box-and-arrow schematics lend themselves well to making connections across the sciences as well as with other disciplines.

The model is a "work in progress" since several relationships require refinement; notably validation and sensitivity analysis of the linkage between carbon and nitrogen dynamics. At present, the model links these two via "plant biomass" and "animal N." We anticipate testing other linkages in the future.

Student Participation

Students selected a component or state variable in the model and investigated the flows in and out of that component. They worked in pairs and

1) used the literature to assess the validity of the component and its dynamics;
2) assessed the sensitivity of the component to alterations in flows;
3) used STELLA® to generate a series of graphs illustrating the consequences of the sensitivity analysis;
4) related the investigation of their component to the work of others in the large group setting; and
5) developed a synthesis paper that critiques the model.

Table 1. Examples of the difference equations and documentation that are developed using STELLA®. Functions such as "NORMAL" are built-in to the program. The timestep (dt) is specified by the modeler and reflects the iterations of the equation or calculation during each time interval. The time interval is also designated by the modeler (e.g., weeks, months, years).

Organic_Matter(t) = Organic_Matter(t-dt) + (Litter_Reduction - Respiration) * dt
INIT Organic_Matter = 5000

DOCUMENT: Organic matter is 5000 g/m^2 and is 10X plant biomass based on several different studies indicating most organic carbon is associated with below ground processes in grasslands. Further, most is associated with organic material that cannot be distinguished as either plant or animal tissue (Clark, 1977).

INFLOWS: Litter_Reduction = Plant_Litter * Temperature * Litter_Water * 0.25 * Fire2

OUTFLOWS: Respiration = Plant_Litter * 0.10

Plant_Biomass(t) = Plant_Biomass(t - dt) + (Leaf_Production + Decreaser + Increaser - Litterfall) * dt
INIT Plant_Biomass = Introduced_Plts + Native_Plts

DOCUMENT: Plant_Biomass is considered to be an estimated standing crop measurement; 500 g/m^2. This is at the high end for a productive mixed and perhaps tallgrass prairie situation (French, 1979).

INFLOWS: Leaf_Production = Table_Leaf
DOCUMENT: Leaf production is a normal function of seasonal plant growth and read in on a monthly basis from Table Leaf.

Decreaser = GRAPH(800)		Increaser = GRAPH(100)	
0, 0.195	7, 0.885	0, 0.280	7, 0.90
1, 0.360	8, 0.835	1, 0.385	8, 0.83
2, 0.420	9, 0.740	2, 0.420	9, 0.72
3, 0.600	10, 0.65	3, 0.605	10, 0.65
4, 0.695	11, 0.58	4, 0.695	11, 0.54
5, 0.895	12, 0.41	5, 0.765	12, 0.415
6, 0.915		6, 0.850	

OUTFLOWS: Litterfall = Plant_Biomass * Fire
DOCUMENT: Litter fall (or accumulation) depends on how much plant biomass is present and it is assumed that 0.25 of the live green plant material becomes litter during the course of the year.

Plant_Litter(t) = Plant_Litter(t - dt) + (Litterfall_Reduction) * dt
INIT: Plant_Litter = 2000

DOCUMENT: Plant litter; 2000 g/m^2 is estimated at four times the live green plant material because; 1) present year's growth (recent standing dead), 2) last year's growth, and 3) even two year old litter contribute to the plant litter compartment. Partial literature support for tallgrass prairie conditions in Schimel (198?). Need information on C/N ratios to accurately predict mineralization and immobilization.

INFLOWS: Litterfall = Plant_Biomass * Fire
DOCUMENT: Litter fall or accumulation) depends on how much plant biomass is present and it is assumed that 0.25 of the live green plant material becomes litter during the course of the year.

Students were introduced to STELLA® and its operation during the first two weeks of the spring semester, 1994. In addition to consideration of the conceptual model (Figure 1), students became familiar with the form of the difference equations used to build the model (Table 1). The students were allowed several weeks to work on the modeling project. Our intent was to allow them to establish ownership and consequently, a sense of responsibility for operation of "their" portion of the model. Their work and effort influenced that of others when the results of the several small groups were brought together.

Student Responses

The results of the student efforts are reported as responses to a learning questionnaire designed to provide feedback on the project (Table 2).

Students generally had minimal to moderate computer experience with most of the experience focusing on word processing. STELLA® was typically considered somewhat problematic. Use of the literature was essential for success in this project, yet nearly 70% of the students reported only low to moderate success in accessing relevant references. Responses in the detailed portion of the evaluation (question #5) revealed that students learned about the simulation process and grassland ecology. Perhaps the most telling question for us was, "What did you learn from this exercise?" Table 3 lists representative comments excerpted from the questionnaire. The unifying lesson students seemed to comprehend was the notion of interaction and interdependence and associated repercussions in ecosystems.

Student success however, was variable. We suggest the importance of:

1) highlighting connections across the science disciplines to nurture creativity;
2) enhancing computer accessibility for a variety of critical thinking activities; and
3) working with students in an inquiry mode.

Our last conclusion underscores the importance of encouraging an intuitive rather than mechanistic approach to biological problem solving. The former is innovative and creative while the latter presumes an understanding of the "parts" before they are arranged to define the "whole." However, the problem is that we rarely know what all the parts are in many biological systems, let alone how they work. As a consequence, our wait before defining the whole may be interminable! Many of our students were reluctant to wade in and "experience" the model because they lacked confidence regarding how certain processes worked; for example, primary productivity, oxidation of organic matter, nitrogen volatilization, etc. If we commit to only *covering* information in biology, we miss great opportunities to *uncover* processes, trends and patterns that make the study of life so fascinating.

We continue to employ computers, particularly in modeling, as an important tool for developing biology graduates who are good thinkers and wise decision-makers.

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Table 2. Evaluation given to students in Biology 307 (Ecology) following completion of the modeling activity.

Computer Simulation Evaluation
Ecology - Spring 1994

Statement of objectives for this exercise:

1. To allow you to become familiar with computers and their use in ecology.
2. To introduce you to the process of computer simulation development in ecology.
3. To teach you about the prairie ecosystem and the nitrogen cycle.
4. To make you aware of the importance of interactions in ecosystem functioning. (You can't change just one thing! One of Barry Commoner's Laws of Ecology.)
5. To give you an appreciation for the complexity in nature, and how difficult it is to manage an ecosystem with human's meager knowledge of how it functions.

Your evaluation (to be done outside of class):

1. How much experience had you had with computers prior to this semester?

Expert	0
A lot	1
Moderate	6
Minimal	21
None	1

2. If you had some experience, please tell what kinds of computers, kinds of software, and what use you were making of them.

IBM	18	Use:	
MAC	15	word processing	19
		video games	6
		spreadsheets	4
		stats/graphics	2
		programming	2

3. To what extent, if any, was the computer or Stella software a handicap to learning about modeling and prairie ecosystems in this exercise? Elaborate, please.

STELLA® as a handicap:	yes	2	
	somewhat	22	
	no	5	
	other	1	(computer center, etc.)

4. How much success did you have in finding information about your component in the library? Explain any difficulties.

High success	9
Moderate success	7
Low success	13

5. On the back of this sheet (additional sheets OK) write a detailed evaluation of this exercise (in reference to objectives above). PLEASE INCLUDE:
   a. Detail what, if anything, you learned from this exercise.
   b. Could this information or technique have been learned without the use of computer simulation?
   c. Explain how this exercise could be improved (assuming we use it in future semesters in Ecology)?
   d. What do you think the benefits or usefulness of computer modeling of ecosystems may be?
   e. What limitations do you see in the use of computer models to predict changes in ecosystems?
   f. Was the model too complex? Too simple? Explain.
   g. Was the model realistic? Explain and give examples.

Table 3. Quoted excerpts of student responses to the question, "What did you learn from this exercise?"

"I learned that the prairie depends upon many factors for good health. When one factor is changed, it will affect the rest of the factors to some extent."

"This exercise allowed me to see things such as how a certain piece of a puzzle will affect the whole puzzle in the end, good or bad or not at all."

"I think computer models make good tools. . .for getting a good idea as to how ecosystems work."

"I learned mainly that there are many interactions in an ecosystem. You cannot alter one aspect and expect all others to remain the same."

"I learned how something that may appear really insignificant to ecological function really will have a great impact somewhere along the line."

"This exercise emphasizes the idea that if only one component of a community is changed many or all of the other components will be affected in some way whether they are organisms or processes."

"I was able to see just how complex and sensitive a prairie is and found it very interesting to be given the ability to change various components within it to see the results it creates. One never really thinks about how rain or temperature affects the environment and through this experiment I was able to actually see how things may possibly react giving me a better understanding of how the environment works and making me more aware of how sensitive the world is."

Loras College, Iowa's oldest institution of higher learning, sits atop the highest bluff in historic Dubuque, overlooking the Mississippi River at the junction of the states of Iowa, Illinois and Wisconsin. Founded in 1839, Loras is a Catholic liberal arts college dedicated to the development of the total student. Small enough to be personal yet large enough to offer a wide variety of quality academic programs, Loras strives to develop students who are active learners, reflective thinkers, ethical decision makers and responsible contributors in their diverse professional, social and religious roles. With an enrollment of approximately 1900 students, Loras offers over 46 majors in five academic divisions.

Our meeting will feature field trips with local experts to a rare blufftop goat prairie, the backwaters of the Mississippi River, the blufftop hardwood forest habitat and historic Galena, Illinois. Dubuque is about a three and one half hour drive from Chicago, Des Moines or Milwaukee, or it can be reached by air via American Eagle from Chicago or Northwest Airlink from Minneapolis. Ethnic and traditional restaurants, a hometown brewery, an opera house, art galleries, antique shops, a river museum and several unique natural areas await your visit to Dubuque during the **1996 AMCBT Meeting, September 19-21, at Loras College.**

The Biology Department at Loras houses the world's largest culture collection of freshwater diatoms. These diatoms, which have recently been aboard two space shuttle flights, are in constant demand by scientists around the world. An NIH and NSF sponsored research colony of axolotl salamanders used in developmental brain research, a vertebrate museum collection and a choice of five off campus field ecology courses are some of the unique components that enhance the undergraduate educational experience in biology at Loras.

The computer facilities at Loras are extensive and user friendly. The campus is connected to the Internet. Currently all of the 60 IBM PS/2 students and faculty computers, as well as all faculty Macintosh computers on campus, are connected to the Internet. The 40 Power Macintosh student computers are networked to local servers for group workshops.

SIDEROPHORES

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When many microorganisms grow in environments limited in the amount of iron, they produce low molecular weight, highly specific iron chelating compounds called siderophores. The presence of this ability is a selective advantage for microbes in such environments. Microorganisms growing within a host organism must also compete with the host's ability to scavenge iron. Therefore, the presence of siderophores contributes to virulence properties of certain pathogenic microorganisms.

The importance of iron as a cofactor cannot be overemphasized. Some essential iron-containing substances include nitrogenase (in nitrogen fixation), ferredoxin, hydrogenase, and cytochromes (in electron transport). Furthermore, iron in excess can generate oxidizing radicals which can be harmful to cells. Iron can promote hydroxyl radical production from superoxide and peroxide, both of which arise from partial reduction of molecular oxygen.

There are a variety of chemically distinct siderophores, but most of them are classified in two chemical groups: phenolates and hydroxamates (see Figure 1). A phenolate example is enterobactin, the siderophore formed by practically all enteric bacteria. Although a number of hydroxamate types have been isolated as products of fungi, there is increasing evidence that these types are present in bacteria. Such types include terregens factor and arthrobactin (*Arthrobacter* species), schizokinen (*Bacillus megaterium* and *Anabaena* sp.) and aerobactin (*Aerobacter aerogenes* and *E. coli* pColV-K30). Citric acid is a siderophore in *Bradyrhizobium japonicum*.

Siderophores can be identified by their unique iron-chelating properties. A highly sensitive method was developed by Schwyn and Neilands (1987). A strong ligand (e.g., a siderophore) is added to a highly colored iron dye complex. The formation of the iron ligand is accompanied by the release of free dye. The free dye has a distinctly different color change. Since the dye which is used is chrome azurol S, the abbreviation for this method is usually CAS. Among other detection methods are visualization by fluorescence under UV light and spraying with 1% (w/v) ferric chloride. The CAS siderophore detection method can be used as a spray or within agar medium.

Experiments

The purpose of these experiments is to isolate and detect siderophores produced by bacteria or fungi. Methods will be presented for isolation, purification, separation and detection.

In addition, extracellular products other than siderophores will be detected. These include amino acids, short chain fatty acids, other lipids and unidentified compounds which are fluorescent in ultraviolet light.

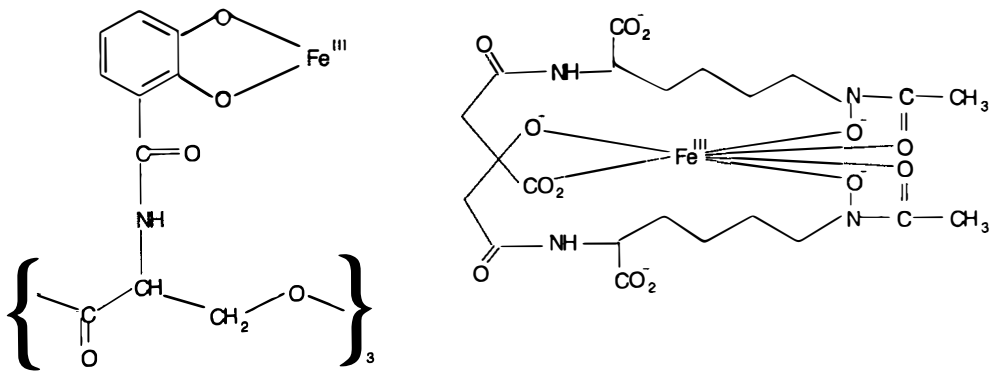

Figure 1. A. Enterobactin, a phenolate. The brackets indicate attachment to cell polymer materials of unspecified length. B. Aerobactin, a hydroxamate.

Methods Used

These laboratories give student experiences with the following technologies:

Growing bacteria
Growing fungi
Replacement cultures of fungi
Adsorption chromatography
Concentration of low molecular weight substances
Paper electrophoresis
Qualitative identification of small molecular weight compounds

Materials

CAS - chrome azurol S (Fluka)
CAS spray - prepared by the instructor and used only in a hood by the instructor
citric acid
ethanol
spraying device
1% (w/v) ferric chloride
ferric chloride spray
HDTMA (Sigma)
M9 minimal medium
methanol
malic acid (optional)
piperazine
potassium phosphate
ninhydrin
Nitrilotriacetic acid
Chelex 100 column
Centrifuge
electrophoresis equipment
(Note to instructors: You may purchase a high voltage unit from Beckmann Instruments. Alternatively, a horizontal gel unit may be modified by insulating spacer bars with tape and draping the paper over the gel pouring chamber, placing the plastic spacer unit in the center. Dimensions of the paper which are given here are suitable for the Horizon 11.14 model horizontal gel electrophoresis chamber from Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD 28877.)
pH meter
spectrophotometer
XAD-7 absorption chromatography resin
0.5 x 10 cm. column
Whatman #1 chromatography paper

Bacterial siderophores

1. Grow bacteria in M9 minimal medium containing the nonassimilable iron chelator nitrilotriacetic acid at 100 micromolar during preparation in order to remove trace contaminating metals. Why was this done?
2. At first, grow 200 mL of a Gram negative bacterium overnight with aeration (in the shaker incubator) at 30°C.
3. Transfer this 200 mL culture aseptically to a 1 liter culture container filed with M9 salts medium and chelator. Aerate the culture for 48 hours with aeration.
4. Centrifuge the culture at 7,000 x g for 15 minutes to remove the cells. The supernatant may be stored at -20°C in a Teflon bottle at this point.
5. Test 1 mL of the supernatant for catecholate siderophore by mixing it with 0.005 mL of 1% (w/v) ferric chloride. A blue-purple color indicates the presence of this type of siderophore. Be sure to use some uninoculated medium as a control.
6. If you have frozen the supernatant, thaw it completely under a running tap.
7. Neutralize the supernatant to pH 7.0: Obtain the pH. If acidic, add 1 M NaOH; if alkaline, add 1 M HCl to neutrality. This will prevent low molecular weight organic acids from adsorbing along with siderophores or other similar compounds in the adsorption chromatography step.
8. Prepare a 0.5 by 10 cm. column of XAD-7, an adsorption chromatography resin.
9. Add the supernatant to the column and let it all flow through it. Collect the eluate and save it in the freezer in a Teflon bottle. Rinse the column with two void volumes (the volume of the liquid phase in a column used for chromatography (Boyer, R., 1986) of deionized water. (What is the void volume? How can you calculate it? Hint: Find the volume of a cylinder which is 5 by 10 cm.)

10. Add one void volume of 1:2 methanol-water mixture. Save the eluate.
11. Add one void volume of 2:1 methanol-water mixture. Save the eluate.
12. Rinse the column with 2 or 3 void volumes of pure methanol. This should contain siderophore, if present.
13. Concentrate the methanol under reduced pressure, if possible. Siderophores are often sensitive to destruction by oxidation, so prolonged exposure to air should be avoided. However, should you not be able to evaporate in the absence of air, loss of siderophore may occur. Other products may be present and studied, however.
14. Resuspend the residue in 0.5 mL of methanol. Put the suspension in a small plastic tube, label and store in the
freezer.
15. Prepare Whatman #1 paper for electrophoresis by cutting a 10x26 cm. piece. Mark the origin line-lightly with a pencil.
16. Spot 10 microliter samples with drying at points on the origin line.
17. 1 microliter samples of 1M citric acid, 1M malic acid and 1M potassium phosphate were spotted as reference standards on the origin line.
18. Subject the samples to electrophoresis in water-acetic acid-formic acid (451:12.5:36) at 500 V for 30 minutes (minimum time).
19. After carefully removing the paper from the electrophoresis unit, hang it in a hood to dry.
20. First, examine the electropherogram under ultraviolet light. Carefully circle any fluorescent spots, and record this information in your notebook.
21. Using a ruler mark off strips from the electropherogram which correspond to separation of different samples. Identical samples can be tested for different substances by using specific sprays, or they can be eluted from the paper with methanol and tested for biological activities.
22. Spray one strip and the controls with CAS spray.
23. Record the distance migrated toward the positive or negative poles for control substances and all observed spots.

Fungi

1. Inoculate a 250 mL erlenmeyer flask containing 100 Sabouraud's dextrose broth with spores and mycelia of a fungus maintained on an agar slant or plate. Aseptically transfer a pea-size bit of growth from the slant to the broth. Incubate at room temperature.
2. After 5 days, examine the fungal cultures. When a pad of mycelium has formed (usually in about 5 to 7 days), the culture is ready for replacing the nutrients ("replacement culture").
3. Carefully decant the nutrient solution with minimal disturbance to the mycelial pad. Rinse the pads with sterile deionized distilled water until no noticeable coloration of the rinse water is seen.
4. Replace each pad with 100 ml of sterile nutrient solution previously adjusted to pH 7. For example, a solution of 1 mg/ml of amino acid (examples: glutamic acid, phenylalanine) can be prepared in advance and sterilized.
5. After 5 days, decant the replacement nutrient solution and save it. If further work is not to commence immediately, refrigerate the solution. If a delay of more than a few days is anticipated, the solution should be frozen.
6. Pour the solution through an XAD7 absorption column. Rinse with 100 ml deionized water. Next, add 40 ml of 1:2 methanol:water solution, saving this and all other fractions. Follow this with a 2:1 methanol:water solution, and finally, rinse with methanol (20 ml).
7. Although all solutions are saved, use only the methanol extract from the absorption chromatography column for further work. Concentrate the methanol by evaporation in the absence of air (rotary evaporator). This should not require much time.
8. Use the concentrated methanol extract for separation by paper electrophoresis as described in the previous section.
9. Complete your analysis for siderophores as described previously.

Universal method to detect siderophores (CAS method)

Ref.—Schwyn, B. and Neilands, J.B. 1987. "Universal chemical assay for the detection and determination of siderophores," *Biochemistry 160*: 47-56.

1. HDTMA (hexadecyltrimethylammonium bromide), purchased from Sigma Corp., is prepared as a 10 mM solution, 6 ml of which is placed in a 100 ml volumetric flask.
2. Iron(III) solution is prepared: 1mM $FeCl_36H_20$, 10 mM HCl
3. CAS (chrome azurol S), purchased from Fluka Chemical Corp., is prepared as a 2mM aqueous solution.
4. Add 1.5 ml of iron(III) and 7.5 ml CAS solutions together, then add them to the 100 ml flask containing HDTMA. Add carefully with stirring.
5. Dissolve 4.307 grams of anhydrous piperazine in water.
6. Carefully add 6.25 ml of 12 M HCl to the piperazine solution.
7. This piperazine solution should be a buffer at pH = 5.6.
8. Add this piperazine solution to the 100 ml volumetric flask.
9. Fill to final volume of 100 ml with water.
10. Store in a polyethylene bottle in the dark at room temperature or lower.
11. Pour this solution in a spraying device to use as a means of detecting siderophores.

Non-specific assays for siderophores

Ref.—Holzberg, M. and Artis, W. 1983. "Hydroxamate siderophore production by opportunistic and systemic fungal pathogens," *Inf. Immunity 46*:1134-1139.

1. 50 - 100 microliters of supernatant is mixed with 0.12 M $FeCl_3$ in 0.005 N HCl to a final volume of 900 microliters. Determine absorbance at 425 nm of the yellow-orange color.
2. 50 - 100 microliters of supernatant is mixed with 5 mM $Fe(ClO_4)_3$ in 0.1 N HCl to a final volume of 900 microliters. Determine the absorbance at 425 nm of the yellow-orange color.

Ferric Chloride Spray Reagent

1. For a solution of 3% ferric chloride in 95% ethanol, weigh 3 grams of ferric chloride and dissolve in 100 ml of 95% ethanol.
2. Store at room temperature in a polyethylene bottle.
3. Add to the spray device as needed.

Ninhydrin Spray

1. Weigh 0.25 grams of ninhydrin and dissolve in 100 ml acetone. This is a 0.25% (w/v) spray solution of ninhydrin.
2. Store in a dark bottle at room temperature.

Additional Experiments

Students who identify siderophores by the technologies introduced in these laboratories should be encouraged to perform a search of the literature to obtain additional information about the kinds and types isolated from the particular microorganism(s) used. Compounds which do not bind iron may be isolated. These could be amino acids, small molecular weight organic acids or pigments.

1. Rinse the unknown compound from the electrophoresis paper with methanol. Obtain an absorption spectrum with a UV-VIS spectrophotometer.

2. Test samples of the unknown compound for antibacterial activity. Pipette onto filter paper disks (about 0.5 cm diameter) and placing them onto tryptic soy agar plates seeded with test microorganisms, such as *E. coli* or *Staphlycoccus aureus.* Incubate at 37°C overnight and look for zones of clearing.

methods mentioned (UV light, CAS spray, ferric chloride spray), test with ninhydrin spray. Search the literature for other possible detection sprays which you can use to detect specific types of substances.

Other experiments could examine the production of siderophores or the other compounds which you observe. For example:

a. try different microorganisms.
b. try different replacement solution for fungi.
c. try adding the iron chelator in the replacement solution for fungi.
d. compare nutrients with and without iron supplement for production of siderophores or other compounds when all other conditions are the same.

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Application For Membership