I. Abstract - The information that you provide in this item may be used in general publications as promotional material. This summary should be original, not an excerpt taken directly from the Project Proposal.

Title of Proposal: Molecular evaluation of *mcyB* and PC-IGS genes in wintering cyanobacterial populations in Pawnee and Branched Oak Lakes of Nebraska

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Summary of Scholarship Activity (maximum of 300 words; Times New Roman, 12 pt font):

Cyanobacteria, more commonly known as blue-green algae, are diverse and widely distributed throughout the world's lakes and reservoirs while possessing the capability to produce a wealth of secondary metabolites. Among this assemblage are a variety of toxic and nontoxic compounds. Communities of phytomicrofauna shift during from a dominant cyanobacterial population to another during the winter months. This project will study the communities of toxic cyanobacterial populations throughout the fall and winter months with biweekly sampling to assess the toxic potential, as determined by the number of toxin producing genes via PCR and qPCR, in each of these ecosystems. Each sample will be analyzed for macronutrients such as nitrogen and phosphorus and trace metals such as zinc and iron to assess correlations between these elemental contributors. Other environmental parameters assessed include temperature, water turbidity, pH, and light intensity.

II. Project Proposal

Title of Proposal: Molecular evaluation of *mcyB* and PC-IGS genes in wintering cyanobacterial populations in Pawnee and Branched Oak Lakes of Nebraska

Amount Requested: $1,577.74

(If more than one student is involved, please provide the above personal information for each student.)

Please respond to each of the following elements in the order presented. Total length of Project Proposal may not exceed five pages. Use Times New Roman, 12-pt. font, with 1" margins.

1. Describe your scholarship or research activity and the specific objectives, or outcomes.

2. Describe your academic preparation for pursuing this activity.

3. Describe how you intend to accomplish your project, the project steps and timeline, the method(s) chosen and how they are appropriate for the discipline. Explain the feasibility of your activity. (Consider time and funding restraints, as well as other factors.) If more than one student is involved please describe exactly what each student will do.

4. Describe the role of your faculty mentor or faculty collaborator. How will s/he be involved in your activity?

5. Describe your plan for a discipline appropriate peer-review of your scholarship or research (for example, journal publication, poster presentation, juried competition and/or exhibition).

6. Explain your budget, describing each line item (justification, the basis of the cost, why needed). Identify any costs allocated directly to faculty (e.g. travel). For Section III of this application use the accompanying form, **Proposal Budget**, to itemize expenses. For both the narrative and the itemized budget, be sure to consider the following:
    a. Equipment: e.g. camera, mazes for mice
    b. Supplies: e.g. chemicals, culture & media, resin, paper, CDs, DVDs, art supplies; remember to include items needed for your presentation (e.g. copy costs, poster materials)
    c. Travel: This can include living expenses for scholarship or research completed during Winter term or in the summer. Personal vehicle mileage should be calculated at .585 cents/mile.
    d. Other: e.g. mice and husbandry costs, theatre props, survey instruments, payment for services
    e. Faculty expenses

    All expenses need to be documented by supplying a copy of the item with a price (for example, a copy of a website order form). If your request exceeds $1,750, please identify priority needs.

RESEARCH/OUTCOMES

Cyanobacteria, more commonly known as blue-green algae, are diverse and widely distributed throughout the world's lakes and reservoirs while possessing the capability to produce a wealth of secondary metabolites. Among this assemblage are a variety of toxic and nontoxic compounds synthesized nonribosomally that includes the neurotoxic anatoxins and hepatotoxic microcystins and cylindrospermopsin (Carmichael, 1994). These toxic compounds have been shown to be detrimental to human and animal health since the first documented case in 1878 in Europe. Colony forming *Microcystis* is the most common microcystin producer found in the freshwater ecosystems of North America (Brittain et al., 2000), however, other organisms such as the filamentous *Planktothrix* and *Anabaena* also possess genes from the *mcy* gene complex that are responsible for the biosynthesis of microcystin. There are over 85 isotypes or congeners of microcystin that can be produced in conjugation with each other and with other toxins such as anatoxin.

In most cases, eutrophication of watersheds with high concentrations of nitrogen and phosphorus produce harmful algal blooms that typically occur during the late summer and early fall and are responsible for the closure of water bodies due to an eminent health risk. It has been estimated that 25 to 75% of all cyanobacterial blooms are toxic (Chorus 2001). In most cases, communities of microfauna shift from one cyanobacterial population to another during the winter months. It has been hypothesized that this shift is from a toxic to a nontoxic community however, historical data suggests that Nebraskan reservoirs, such as Pawnee and Branched Oak Lakes, possess communities that retain the ability to produce microcystin throughout the winter months.

This project will study the communities of toxic cyanobacterial populations throughout the fall and winter months to assess the toxic potential, as determined by the number of toxin producing genes, in each of these ecosystems. Regular, biweekly sampling will measure the number of *mcyB* genes*,* one of the most variable *mcy* gene sequences within the genome, and the phycocyanin intergenetic spacer region, PC-IGS, a photosynthetic pigment common to all freshwater cyanobacteria, to quantify the number of cyanobacteria present in each week's sample. Sampling will continue through March of 2009 with the completion of all sample analysis by mid April.

ACADEMIC PREPARATION

Over the past three years, I have been involved with a variety of independent research projects studying aspects related to the current proposal. In the spring of 2007, I was awarded a Fleming collaborative grant to create a LC/MS standard of a rare neurotoxin, known as anatoxin-a(s), produced by certain species of cyanobacteria, particularly *Anabaena*. Following this experience, I was accepted into a two year research program by the National Institutes of Health as part of the IDea Network for Biomedical Research Excellence. My first year was spent working at the University of Nebraska-Lincoln studying Chlorella viruses, a green algal virus, from the alkali lakes in West Central Nebraska. The past summer, I was awarded an internship with the National Oceanic and Atmospheric Administration at the Great Lakes Environmental Research Laboratory in Ann Arbor, MI. Not only do I have research preparation, but I have taken courses to emphasize environmental and natural sciences such as ecology, natural science, tropical biology, etc. with adequate field research techniques. Please see my attached CV (Appendix C) for a complete listing of research experience and relevant presentations

METHODOLOGY

Location

Two freshwater reservoirs, Pawnee and Branched Oak Lakes, have been selected for their consistently high summer concentrations of microcystin over the past four years. Two locations within each lake were marked with buoys where depths exceeded 20 ft.

Sample collection

Regular, biweekly samples will be taken one meter below the surface and one meter above the bottom with the use of a kemmer bottle at the two marked locations in each lake through 15 March 2009. Approximately one liter of water will be collected from each depth and 100 mL will be preserved with 1% lugol's preservative.

Sample Analysis

Each sample will be analyzed for total nitrate (NO_3) and total phosphorus in the field. In the lab, 100 mL will be filtered onto 0.8 μm pore size filters to collect and concentrate cyanobacteria. Cell counts and species counts will be tabulated from the 100 mL of preserved sample.

DNA extraction

Following filtration, samples will undergo an extraction procedure to remove the cyanobacterial DNA using a predetermined protocol (appendix A). Some experimentation with protocol and culture samples will be necessary. In order to accomplish this, cultures will be filtered and subjected to three protocols to establish which methods frees the DNA with the most efficacy.

PCR

PCR with the PC-IGS primers will be used to confirm the presence of cyanobacterial DNA in the sample. qPCR with primers for *mcyB,* specific to *Microcystis* and PC-IGS will be used to quantitate the number of toxin genes and phycocyanin genes in each sample. This number is related to the total number of cyanobacteria in the sample. Protocol is attached in appendix A)

FACULTY MENTOR

Dr. Jerald Bricker, Associate Professor of Biology, will act as my mentor for this project. He will aid in instrumentation and field sampling techniques as well as provide assistance for sampling when needed.

PEER-REVIEW

The data collected from this research will be presented at the Nebraska Academy of Sciences in April as well as at the West Coast Biological Sciences Undergraduate Research Conference in San Diego, CA.

LITERATURE CITED

Carmichael, W. W. 1994. The toxins of cyanobacteria. Sci. Am. 270:78-86.

Chorus, I. 2001. Cyanotoxin occurrence in freshwaters—A summary of survey results from different countries. In *Cyanotoxins—Occurrence, causes, consequences,* ed. K Chorus, pp. 75-78. Berlin: Springer-Verlag

III. Proposal Budget

Title of Proposal: Molecular evaluation of *mcyB* and PC-IGS genes in wintering cyanobacterial populations in Pawnee and Branched Oak Lakes of Nebraska

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Part II, the Project Proposal must include a budget justification describing each of these line items, the basis of the cost, and why it is needed. Identify any costs associated directly with the faculty collaborator/mentor (e.g. travel). All expenses should have documentation supporting the cost (supply a copy of a price list or website order form, etc). If your request exceeds $1,750, please identify priority needs.

Note: This application is for Scholarship and Research Grants. If your proposal is for presentation expenses only, please complete the Presentation Award application.

Period of Performance: **Sept 2008** (start date) **May 2009** (end date)

Description (include tax and shipping costs)		Cost per unit	Qty.	Amount Requested
1. Equipment				
Provided through Biology Department				NA
2. Supplies				
laboratory supplies (microfuge tubes, centrifuge tubes, etc.)		$200.00		$200.00
DNeasy plant mini kit (50)		$169.00	2	$338.00
Glass beads, acid-washed		$87.85	1	$87.85
DNAzol ES		$37.00	3	$111.00
Marker HAE III OX174 500g		$184.41	1	$184.41
Hardware for buoy construction (foam, hardware, etc.)		$55.66		$55.66
Filter supr800 .8um 47mm		$120.72	2	$241.44
PCR primers		$300.00		$300.00
3. Travel				
Gas		2.75/gal	20.17	$55.46
personal vehicle use		0.585	605	$353.93
Personal Vehicle mph	~30			
miles per sampling trip	55			
No. of sampling trips	11			
5. Other				NA
6. Faculty Stipend				$250.00
7. INBRE Grant (subtracted from total)				$600.00
			Total	$1,577.74

A complete list of ordering information can be found in appendix B.

Appendix A.
STANDARD PCR PROCEDURE FOR ENVIRONMENTAL SAMPLES

PRESTART CHECKLIST

-remove samples from DNA freezer in PCR room to thaw in DNA room
-place sterile dH_2O and master mix microfuge tubes into Clone zone and turn on UV for 30 minutes
-turn on thermocycler with prescribed cycle settings
-wipe down entire area with 70% EtOH

RECORD SAMPLES, MASTER MIX RECIPE AND THERMOCYCLER CYCLES IN NOTEBOOK

- should have a negative PCR control (all master mix components + dH_2O)

Sample description (today's date, sampling trip, sample data, type of sample (e.g. extracted 5.28.08), type of procedure (e.g. PCR *mcy B*)
Sample list
1. ID numbers
2. Extraction control
3. NR - PCR control (master mix + dH_2O)
Master Mix Recipe
May need to add BSA or DMSO to bind other compounds
Cycle – use robocycler and set cycle times
Gel – 1% agarose, 100 V, 30 min
Photograph with labeled lanes
Results/ Conclusions/summary

PREPARE MASTER MIX IN CLONE ZONE

- recipes are usually similar with the exception of primers
- primers for *mcy B* or PC-IGS (phycocyanin innergenic space) may be used
- add all components except platinum DNA Taq pol (temperature sensitive- remove from freezer only after all others have been added and return immediately)
- vortex all components prior to adding. Do not vortex *Taq*
- always use stuff tips for PCR preps
- add 48 µL to each prelabeled PCR tube in Clone Zone
- transfer PCR tubes to outside rack
- general rule: use 2µL DNA from environmental samples and 1µL DNA for culture samples

TABLE 2.1: MASTER MIX RECIPE EXAMPLE

COMPONMENTS	1X VOLUME IN EACH TUBE	7.5X TOTAL VOLUME NECESSARY FOR ALL SAMPLES PLUS 0.5 EXTRA
10 X BUFFER	5 µL	37.5 µL
50mM $MgCl_2$	2.5	18.75
dNTP	1	7.5
mcy B F primer (or PC β F) with conc. and date	0.7	5.25
mcy B R primer (or PC α R) with conc. and date	0.7	5.25
dH_2O	37.9	284.25
plat *Taq* polymerase	0.2	1.5
DNA (from environmental samples)	2	
TOTAL	50	

**ADD DNA TO EACH SAMPLE IN OTHER ROOM, WATER FOR NR*

- tubes can be left open until DNA has been added
-

**PLACE SAMPLES IN THERMOCYCLER*

- Try to avoid placing samples around the parameter of the block, temperature may not be constant.
- Also try to avoid leaving samples in the cycler over night- this is a possibility, but don't do it all the time.

TABLE 2.2: THERMOCYCLER CYCLE EXAMPLE

TEMPERATURE	TIME (MINUTES)		
94º C INITIAL DENATURE	5		
94º C DENATURE STEP	1	30X	Temp of annealing step may vary due to primers
53º C ANNEALING STEP	1		
72º C ELONGATION STEP	1		
72º C	7		Increase temp = increase primer specificity
6º C STORAGE	∞		Decrease temp = decrease primer specificity

**run 10 µL PCR product with standard on 1% agarose gel at 100 V for 30 minutes*

- use 1X TAE (tris, acetic acid, EDTA) for running buffer, gels and EtBr solution
  - to make new EtBr solution, add 20 mL stock EtBr per 1 L dH_2O
- use 1µL 6X loading dye for 10 µL PCR product (parafilm works great for this)
  - dye has 3 colors: blue, purple, pink
    - purple should migrate same distance as PCR product (~685 bp)
    - pink has primer dimmers formed during reaction
- 1.5 µL ΦX marker standard – always put in first lane of gel
- *Note – increasing agarose concentration will slow the migration of fragments allowing for a clearer separation between bands*

****notes to self**:*

Never touch sample tubes without gloves
Never touch gloves to unsterile surfaces
Always change gloves when reentering PCR room

SEE ATTACHED FOR EXAMPLE OF NOTEBOOK

APPENDIX B
DNA EXTRACTION OF WATER COLUMN SAMPLES ON SUPOR FILTERS

**NO MORE THAN 8 SAMPLES/ DAY INCLUDING A BLANK SHOULD BE PROCESSED*

**PRESTART CHECKLIST*

- turn heat block on to 90º C
- wipe down work area and pipettes with 70% EtOH

DNAZOL:

- label 2 mL orange screw cap tubes with appropriate sample number
- transfer dried filter from original tube with sterile metal forceps found under scale
    - soak forceps in DNA away for 1 min then rinse with dH_2O prior to transferring next sample. Repeat for all samples
- Add 1 mL DNAzol ES (Molecular Research Center, Cincinnati, OH, #DN128) to each tube and mix well
    - It is necessary to have a negative extraction control that contains only DNAzol
- Heat at 90º C for 2-4 hours vortexing every ½ hour for 15-20 seconds
    - At 2 hours:
        - Use a p20 (green box) pipette tip to add ~0.3 g glass beads (150-200µm, washed in nitric acid and autoclaved) to each tube.
        - Beat in bead beater for 3 minutes (18 intervals) at speed 42
        - Let tubes set to settle bubbles. Should have less than half a tube of bubbles before returning to heat
    - At 4 hours:
        - Beat in bead beater for 3 minutes (18 intervals) at speed 42

POSSIBLE STOP POINT

- turn heat block temperature down to 65º C
- In hood, pour chloroform into 50 mL falcon tube and transfer 1 mL to each tube. Then **SHAKE** (not vortex) for 20 seconds. This removes pigments and insoluble plant debris.
- Let tubes sit for 5 minutes then spin at 13.2 rpm for 10 minutes. Take care to place in particular order incase numbers are removed.
    - Label new set of tubes with sample ID numbers
- Transfer upper aqueous layer to new tubes.
- Repeat chloroform extraction. Should do this no more than twice to minimize loss of DNA
- Using p1000 pipette for transfer, measure and record volume of extract. Volume should be under 1 mL. No more than 950 µL of extract should be used in one tube. If more, transfer to 2nd tube.
- Add 0.75X 100% EtOH (molecular biology grade) to each tube to precipitate DNA. Invert to mix. Let sit for 5 minutes then spin for 4 minutes at 6.6 rpm
- Decant supernatant, careful not to touch the rim of the beaker to minimize contamination. Invert tubes on large Chem wipe to dry.
- Resuspend pellet with 150 µL dH_2O using stuffed tips

POSSIBLE STOP POINT

QIAGEN PLANT KIT: ALL PROCEDURES TAKEN FROM KIT BOOKLET
(DNeasy Plant Kit, Valencia, CA, #69104)

- add 400 µL AP1 buffer and 3 µL RNase A (100 mg/mL). RNase does not need to be kept cold.
- Vortex and incubate at 65º C for 10 minutes, vortexing occasionally. May turn cloudy if ethanol is present in tube.
- Add 130 µL AP2 buffer (use 130 µL AP2 per 400 µL total). This should turn cloudy.
- Incubate tubes for 10 minutes in -20º C freezer (freezer in PCR room). This precipitates detergent, proteins and polysaccharides.
    - Heat dH_2O at 65º C. This will be needed later.
    - Label 2 mL orange screw cap tubes with sample numbers
    - Label 1.5 mL snap cap tubes with the following information
        - Cap:
            Sample ID No.
            Sampling Ship Name
            Date sample collected
        - Side:
            "DNA Extract"
            Today's date
- Spin tubes for 15 minutes at 13.2 rpm
- **TRANSFER SUPERNATANT** to new tube making note of volume
- Add 1.5X vol AP3/EtOH buffer. Mix by pipetting.
- Add sample to clear spin column, filling to the edge of the first tube. Spin for 1 minute at 8.0 rpm. **DISCARD FILTRATE**. Repeat as necessary until all sample has been filtered.
- Add 500 µL AW buffer (70% EtOH diluted with TE) and spin for 1 minute at 8 rpm. **DISCARD FILTRATE**. Repeat. May use one pipette tip if tip touches nothing between transfers.
- Spin for 2 minutes at 13.2 rpm to remove all traces of EtOH from column
- Transfer columns to prelabeled 1.5 mL snap cap tubes. Add 50 µL 65º C dH_2O to the center of the column. Let sit for 5 minutes then spin for 1 minute at 8 rpm
- Store extracts at -20º C
- Wipe down pipettes/ work space with 70% EtOH