Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2014 Work Plan

Date of Report: January 10, 2014

Date of Next Status Update Report: January 1, 2015

Date of Work Plan Approval:

Project Completion Date: June 30 , 2017

Does this submission include an amendment request? No

PROJECT TITLE: Antibiotics and antibiotic resistance genes in Minnesota lakes

Project Manager: William Arnold

Organization: University of Minnesota

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Location: Statewide

Total ENRTF Project Budget: $300,000	**ENRTF Appropriation:**	**$300,000**
	Amount Spent:	**$0**
	Balance:	**$300,000**

Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03e

Appropriation Language:
$300,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to quantify the relationship between antibiotics and antibiotic-resistant bacteria in Minnesota lakes to determine if improved wastewater treatment is necessary to protect human and aquatic health. This appropriation is available until June 30, 2017, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Antibiotics and antibiotic resistance genes in Minnesota lakes

II. PROJECT STATEMENT:
Pharmaceuticals are found in water bodies all across Minnesota. These compounds are biologically active and can disrupt the function of ecological communities or have other adverse effects. Of particular concern are antibiotics, one of the greatest inventions of the 20th century. The utility of antibiotics is at risk, however, due to resistance in clinical settings. The release of antibiotics and antibiotic resistance genes into the environment may also pose a threat to human health by encouraging broader development of antibiotic resistance or by leading to the harboring of elevated levels antibiotic resistance genes in environmental matrices. There is also potential for antibiotics to disrupt the proper functioning of ecosystems. While there is a background level of naturally occurring antibiotic resistance, elevated or persistent levels due to human activities have the potential to cause harm to human, veterinary, or ecosystem health. The overall goal of this project is to improve water quality and to protect human and ecosystem health by 1) quantifying the current and historical levels of selected human and veterinary antibiotic compounds in lake sediments, and 2) determining the current and historical levels of genes that code for resistance to the selected human and veterinary antibiotics in lake sediments. The results of this work will reveal if the environmental presence of human and veterinary antibiotics in Minnesota lake sediments leads to the retention of resistance genes.

III. PROJECT STATUS UPDATES:

Project Status as of January 1, 2015:

Project Status as of July 1, 2015:

Project Status as of January 1, 2016:

Project Status as of July 1, 2016:

Project Status as of January 1, 2017:

Overall Project Outcomes and Results:

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collection and dating of sediment cores
Description: Based on our previous ENTRF sponsored work, we have identified three wastewater impacted sites (Lake Pepin, Duluth Harbor, and Lake Winona) for study. Both Duluth Harbor and Lake Winona directly receive wastewater effluent. Lake Pepin (a natural "lake" within the Mississippi River) receives some effluent directly, but its watershed covers two-thirds of the state of Minnesota, so it serves as an integrative site. To complement the samples from these sites, we will also collect surface sediment samples behind Ford Dam in St. Paul (just upstream of the confluence of the Minnesota and Mississippi Rivers) and from Rice Lake in Brainerd. These latter two samples will help us parse out the effects of large fractions of the State's watershed. The control site will be Little Wilson Lake, which has no wastewater input.

Cores will be collected by a piston or box-type corer. Riverine surface sediment samples will be collected with a dredge or scoop, depending on the depth. The cores will be extruded in the field in 1 to 4 cm sections with subsamples being taken for dating and determination of resistance gene levels. The remainder of the sample will be dedicated to chemical analyses. The Lake Pepin core will be dated via magnetic susceptibility, and it will be sectioned in the laboratory after dating is performed. The other cores will be dated using lead-210 and cesium-137 methods and other chemical markers as described in Dr. Engstrom's recent work. We will collect cores that are deep enough (i.e., go back it time far enough) such that we will have core sections that date to prior to the

deployment of the antibiotic classes (1930-1960 depending on the class). The water and organic matter content will be determined as a function of depth via loss on ignition analysis. Because antibiotic resistance levels may be related to heavy metal content, all sediment samples will be analyzed via inductively coupled plasma-mass spectrometry (ICP-MS; Department of Earth Sciences, U of MN) to determine the metal concentrations. Sediment deposition rates as a function of time will be calculated based on the mass of sediment contained between dated points in the core section.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 46,500
Amount Spent: $ 0
Balance: $ 46,500

Activity Completion Date:

Outcome	Completion Date	Budget
1. Core collection	10/30/14	$ 14,500
2. Core dating and determination of organic content and deposition rates	7/1/15	$ 32,000

Activity Status as of January 1, 2015:

Activity Status as of July 1, 2015:

Activity Status as of January 1, 2016:

Activity Status as of July 1, 2016:

Activity Status as of January 1, 2017

Final Report Summary:

ACTIVITY 2:

Activity 2: Measurement of sulfa, tetracycline, macrolide, and quinolone antibiotics as a function of depth/time in sediment cores

Description:. By analyzing the antibiotic concentrations as a function of depth, it will be possible to assess the "dosage" each lake received as a function of time. The trends in antibiotic levels will be related to any trend in resistance determined in Activity 3.

The sediment cores will be sectioned as a function of depth. Wet samples with a mass corresponding to ~10 g dry weight will be freeze dried. The freeze-dried sample will be spiked with ^{13}C-labelled compounds (one for each antibiotic compound class to be studied: sulfonamides, macrolides, fluoroquinolones, and tetracyclines) as isotope dilution internal standards. A single un-spiked blank sample of clean sand will be processed and analyzed to ensure that there is no contamination. A recovery standard (a sediment from depth great enough that it should have minimal antibiotics present) will be spiked with $^{13}C_{12}$-labeled and unlabelled antibiotics to test recovery. The samples will be extracted using an accelerated solvent extraction system. The exact protocol will need to be optimized, but two options are a 50:50 mixture of pH 6 phosphate buffer and methanol or a 75:25 ratio of acetonitrile and water (50-75% recovery in initial tests). The extract is then evaporated to remove the organic solvent, and the water portion is cleaned up and concentrated using pre-washed Oasis HLB solid phase extraction cartridges. After elution in acetonitrile/methanol, the eluate is then concentrated, and solvent exchanged into the appropriate eluent matrix with a volume of 100-200 μL. Note that both the pore water and sediment are extracted, but given the high solid to water ratios, the pollutant levels are attributed to the sediment phase. Analysis of the samples will be performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (available in the U of MN Cancer Center on an hourly basis). From the data derived from analyses above, the concentrations (mass per mass) and accumulation rates

(mass per area per time) of the antibiotics will be calculated. Because clinical use of antibiotics began in the 1930s, sediments deposited prior to this date will serve to reveal and natural background concentrations for those compounds that can be produced naturally (i.e., macrolides and tetracyclines).

Summary Budget Information for Activity 2:

ENRTF Budget: $ 127,000
Amount Spent: $ 0
Balance: $ 127,000

Activity Completion Date:

Outcome	Completion Date	Budget
1. Optimize antibiotic extraction and analytical methods	7/31/15	$ 43,500
2. Measure antibiotic concentrations in sediment samples	12/31/16	$ 73,500
3. Calculate accumulation rates	1/31/17	$ 10,000

Activity Status as of January 1, 2015:

Activity Status as of July 1, 2015:

Activity Status as of January 1, 2016:

Activity Status as of July 1, 2016:

Activity Status as of January 1, 2017

Final Report Summary:

ACTIVITY 3: Measurement of antibiotic resistance genes as a function of depth/time in sediment cores
Description: Antibiotic resistance levels can be measured in sediment samples using techniques developed in previous ENTRF work. Sediment cores will be sectioned as a function of depth in parallel with Activity 2. Genomic DNA will be extracted and purified from these samples and then used as template to genetically determine the amount of antibiotic resistance in these samples. Genomic DNA will be extracted and purified from sediment samples. Briefly, about 500 mg of sediment (wet weight) will be processed using a bead beater to lyse cells. Genomic DNA will be then extracted and purified from sediment samples using a FastDNA Spin Kit for soil (MP Biomedicals; Solon, OH). All genomic DNA extractions will be performed in triplicate and stored at -20ºC until needed. Quantitative real-time PCR (qPCR) will be used to quantify 16S rRNA genes (a measure of total bacterial biomass) as well as three genes encoding tetracycline resistance (*tet*(A), *tet*(W) and *tet*(X)), the integrase gene of class 1 integrons (*intI1*), one gene encoding sulfonamide resistance (*sul1*), and one gene encoding resistance to macrolides (*erm* (B)). These genes will be targeted in this study because these genes encompass a variety of resistance mechanisms as well as resistance genes encoding proteins that act against different classes of antibiotics. The qPCR analysis will be conducted using an Eppendorf Mastercycler ep realplex thermal cycler (Eppendorf; Westbury, NY). Each qPCR run will consist of initial denaturation for 10 min at 95°C, followed by forty cycles of denaturation at 95°C for 15 s, and anneal and extension at 60°C (most targets) or at 56°C (human-specific Bacteroides) for 1 min. A 25 μL reaction mixture contained 12.5 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad; Hercules, Calif.), 25 μg bovine serum albumin (Roche Applied Science; Indianapolis, Ind.), optimized quantities of forward and reverse primers, and a specified volume of template DNA (usually 0.5 μL). The precise volume and concentration of template DNA will be empirically optimized for each sample to generate the lowest detection limit while minimizing inhibition of PCR. The quantity of target DNA in unknown samples will be calculated based on a standard curve generated using known quantities of template DNA. Standards for qPCR have already been prepared by PCR amplification of genes from positive controls, followed by ligation into pGEM-T Easy (Promega; Madison, Wisc.). Ten-fold serial dilutions of plasmid DNA will be prepared and run on the thermal cycler to generate standard curves ($r^2 > 0.99$).

Results will be correlated to sediment age (Activity 1) and to antibiotic levels (Activity 2).

Summary Budget Information for Activity 3:

ENRTF Budget: $ 126,500
Amount Spent: $ 0
Balance: $ 126,500

Activity Completion Date:

Outcome	Completion Date	Budget
1. DNA extraction and purification	5/31/15	$ 30,000
2. Quantify known antibiotic resistance genes	4/30/17	$ 86,500
3. Data synthesis, reporting, and recommendations	6/30/17	$ 10,000

Activity Status as of January 1, 2015:

Activity Status as of July 1, 2015:

Activity Status as of January 1, 2016:

Activity Status as of July 1, 2016:

Activity Status as of January 1, 2017

Final Report Summary:

V. DISSEMINATION:

Description: The results will be disseminated via peer reviewed publications in scientific journals, presentations at local/regional conferences, and via a publically available final report. Partnering with Dr. Engstrom provides additional education and outreach opportunities via the Science Museum of Minnesota.

Status as of January 1, 2015:

Status as of July 1, 2015:

Status as of January 1, 2016:

Status as of July 1, 2016:

Status as of January 1, 2017

Final Report Summary:

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Explanation
Personnel:	$ 250,000	Arnold at 4-6% time per year. LaPara at 1-2% time per year. Graduate students (43-50% time) and/or postdoc (75% time). Costs include fringe benefits for all and tuition for the graduate student.

Professional/Technical/Service Contracts:	$ 16,000	Science Museum of Minnesota and Daniel Engstrom for assistance with core collection and dating.
Equipment/Tools/Supplies:	$ 29,000	Chemical standards, isotope standards, microbiologial/DNA extraction kits, instrument/analytical time for antibiotic and DNA analysis, solvents, consumable supplies, notebooks, software licenses. Equipment maintenance.
Travel Expenses in MN:	$ 5,000	Mileage charges and university vehicle rental charges for trips to collect water samples. Hotel/meal charges if overnight stay required.
TOTAL ENRTF BUDGET:	**$ 300,000**	

Explanation of Use of Classified Staff: not applicable

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 3.5

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 0.1

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$ 125,000	$ 0	Arnold and LaPara will also devote 1% time per year in kind ($10,700). Because the project is overhead free, laboratory space, electricity, and other facilities/administrative costs (52% of direct costs excluding permanent equipment and graduate student academic year fringe benefits) are provided in-kind ($114,300)
State			
	$ 0	$ 0	
TOTAL OTHER FUNDS:	**$ 125,000**	**$ 0**	

VII. PROJECT STRATEGY:

A. Project Partners: : The project will be led by William Arnold and Timothy LaPara (University of Minnesota, Department of Civil Engineering). The team will consist of two graduate student researchers. Dr. Arnold has extensive experience quantifying chemicals in environmental matrices, and Dr. LaPara is an expert on the quantification of resistance genes. Daniel Engstrom at the Science Museum of Minnesota will perform the core collection and dating.

B. Project Impact and Long-term Strategy:

This project will provide an understanding of the historical levels of antibiotics used in human and veterinary medicine that have entered Minnesota lakes. Additionally, this will be the first study to investigate how the discharge of these chemicals has or is affecting the levels of resistance genes in the environment. This is

information critical to protecting human and ecological health and may provide information relevant to antibiotic use and development. This study will reveal if additional treatment to remove antibiotics from wastewater or runoff is necessary or unnecessary in terms of proliferation of resistance genes.

VIII. ACQUISITION/RESTORATION LIST: not applicable

IX. VISUAL ELEMENT or MAP(S): See attached.

X. ACQUISITION/RESTORATION REQUIREMENTS WORKSHEET: not applicable

XI. RESEARCH ADDENDUM: to be inserted upon completion of peer review

XII. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted not later than January 1, 2015; July 1, 2015; January 1, 2016; July 1, 2016, and January 1, 2017. A final report and associated products will be submitted between June 30 and August 15, 2017.

Environment and Natural Resources Trust Fund

M.L. 2014 Project Budget

Project Title: Antibiotics and antibiotic resistance genes in

Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03e

Project Manager: William Arnold

Organization: University of Minnesota

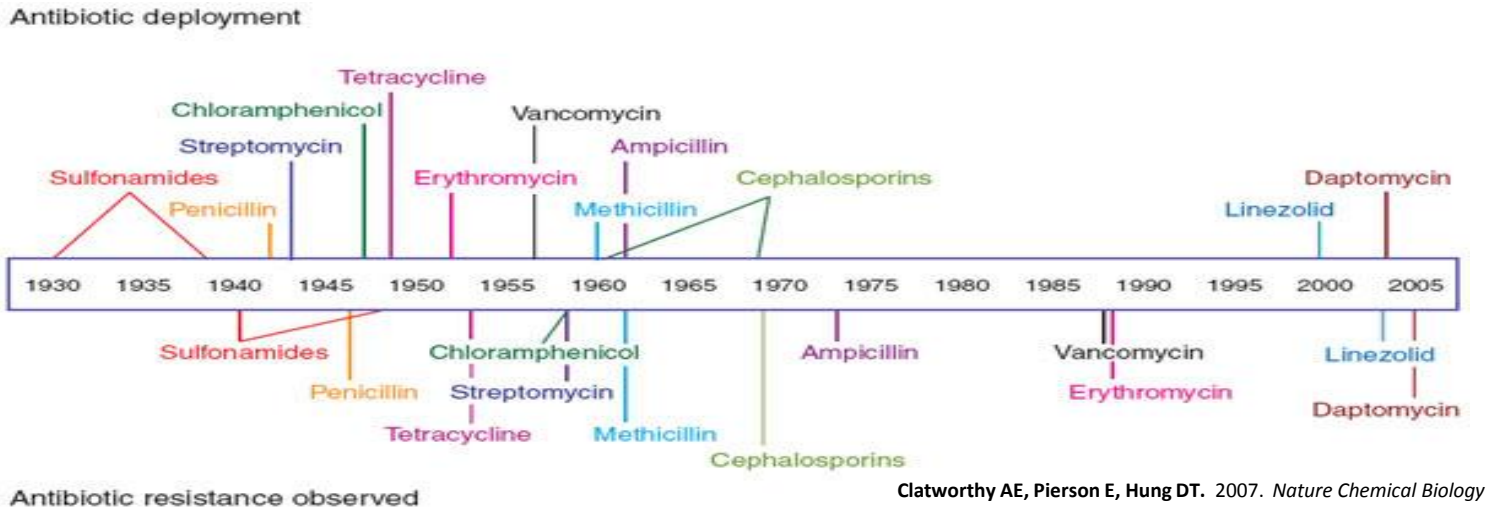
M.L. 2014 ENRTF Appropriation: $ 300,000

Project Length and Completion Date: #3 Years, June 30, 2017

Date of Report: January 10, 2014

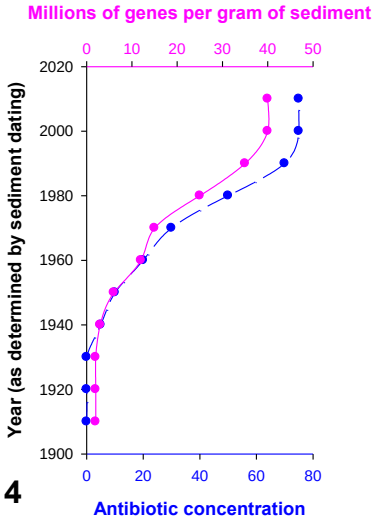
ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	**Collection and dating of sediment cores**			**Measurement of sulfa, tetracycline,**			**Measurement of antibiotic resistance genes**				
Personnel (Wages and Benefits)	$24,500	$0	$24,500	$113,000	$0	$113,000	$112,500	$0	$112,500	$250,000	$250,000
Arnold (PI, 6% time per year Y1 and Y2, 4% Y3. Estimated total: $36,200). Project supervision, supervision of graduate student #2/postdoctoral researcher #1 and project reporting.											
LaPara (co-PI, 2% time per year Y1 and Y2, 1% Y3. Estimated total: $9,600). Project supervision, supervision of graduate student #1 and and project reporting.											
Graduate student #1 (43.75-50% time in Y1 and Y2, 25-50% time in Y3. Estimated total: $122,700). Extraction and purification of DNA from collected sediment samples. Quantificaiton of resistance genes.											
Graduate student #2 (43.75-50% time in Y1 and Y2, 25-50% time in Y3. Estimated total: $81,500) or Postdoctoral Researcher #1 (75% time in Y1 and Y2). Sediment core collection and sectioning. Development of antibiotic extraction and analytical protocols. Determination of antibiotic concentrations in sediments.											
Professional/Technical/Service Contracts											
Science Museum of Minnesota for collection and dating of sediment cores. Costs include personnel (Dr. Daniel Engstrom, 2% effort $4688 salary, $1312 fringe) and analytical and dating costs ($10,000).	$16,000	$0	$16,000							$16,000	$16,000
Equipment/Tools/Supplies											
Supplies including chemical standards, isotope standards, microbiologial/DNA extraction kits, instrument/analytical time for antibiotic and DNA analysis, solvents, consumable supplies, notebooks, software licenses	$1,000	$0	$1,000	$12,000	$0	$12,000	$12,000	$0	$12,000	$25,000	$25,000
Maintenance and repair of laboratory equipment required for analyses and experiments				$2,000	$0	$2,000	$2,000	$0	$2,000	$4,000	$4,000
Travel expenses in Minnesota											
Mileage charges and univeristy vehicle rental charges for trips to collect water samples. Hotel/meal charges if overnight stay required.	$5,000	$0	$5,000							$5,000	$5,000
COLUMN TOTAL	**$46,500**	**$0**	**$46,500**	**$127,000**	**$0**	**$127,000**	**$126,500**	**$0**	**$126,500**	**$300,000**	**$300,000**

The problem: Antibiotic use causes antibiotic resistance



Clatworthy AE, Pierson E, Hung DT. 2007. *Nature Chemical Biology* 3(9):541-548.

The question: Do antibiotics released into aquatic systems lead to the retention or development of resistance?



Benefits

- Evaluation of antibiotic resistance reservoirs in aquatic systems
- Determination of need for additional wastewater treatment
- Protecting human and ecological health
- Provide information relevant to antibiotic use and development