

2014 Project Abstract

For the Period Ending June 30, 2017

PROJECT TITLE: Antibiotics and antibiotic resistance genes in Minnesota lakes

PROJECT MANAGER: William Arnold

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FUNDING SOURCE: Environment and Natural Resources Trust Fund

LEGAL CITATION: M.L. 2014, Chp. 226, Sec. 2, Subd. 03e

APPROPRIATION AMOUNT: \$300,000

AMOUNT SPENT: \$300,000

AMOUNT REMAINING: \$0

Overall Project Outcomes and Results

Antibiotics are substances that stop the growth of or kill bacteria. Animal agriculture and human medicine are the largest consumers of antibiotics worldwide. A fraction of the antibiotic administered is excreted in its original form through urine and/or feces. These residues reach aquatic environments through the discharge of wastewater effluent or drainage and surface runoff from agricultural fields to which manure has been applied. The presence of antibiotics in the environment are of concern, because these chemicals may select for and proliferate the occurrence of antibiotic resistance genes (ARGs). ARGs allow bacteria to survive in the presence of an antibiotic. Heavy metals are also known to co-select for ARGs. The World Health Organization has identified antibiotic resistance as one of the major threats to global health. The increase in the prevalence of antibiotic resistant infections, coupled with the decrease in the development of new antibiotics, emphasize the need for new strategies to better understand antibiotic resistance.

The goal of the project is to quantify the current and historical levels of selected human and veterinary antibiotic compounds and genes that code for their resistance in lake sediments. Sediment cores collected for three anthropogenically-impacted Minnesota lakes (Lake Pepin, Duluth Harbor, and Lake Winona) and a control lake in Superior National Forest (Little Wilson Lake) were radiometrically dated. The twenty antibiotics included in this study have a mixture of human and/or agricultural uses, some are known natural products, and they span several of the major classifications (sulfonamides, fluoroquinolones, tetracyclines, macrolides).

Sediment cores were successful at capturing the usage trends of ten antibiotics. The initial appearance of antibiotics in the sediment core generally agreed with the FDA approval date, which provided further confidence in the dating of the sediment cores and the ability of sediment cores to capture antibiotic usage trends. Ofloxacin, trimethoprim, sulfapyridine, and sulfamethazine were the only antibiotics to be detected in all three anthropogenically-impacted studied lakes with levels up to 91.7, 2.5, 13.1, and 5 ng g⁻¹, respectively. Human-use antibiotics were detected more frequently and at higher concentrations than antibiotics used for veterinary medicine. Also, the degree of antibiotic pollution appeared to be a function of treated wastewater impact. Lake Winona was the most heavily wastewater impacted lake in the study (approximately 63% of the inflow is treated wastewater effluent) and had the highest concentrations and greatest number of antibiotics detected. Treated municipal wastewater is likely the primary contributor to antibiotic pollution in the studied lakes.

The abundance of 48 antibiotic, metal, and antibiotic-associated resistance genes were quantified in the sediment cores with detected levels ranging from 10³ to 10⁸ gene copies per gram. Most ARGs included in this study, however, were not consistently quantifiable throughout the sediment cores.

Similar concentrations of *bla*_{SHV}, *cadA*, *copA*, *int11*, and *mexB* were measured amongst the sediment cores, but Lake Winona had higher levels of *sul3* and *tet(A)* compared to the other lakes. ARGs levels did not appear to be a function of sediment core depth, and thus the measured levels are at or close to natural, indigenous background levels of the studied genes. Also, (unlike the antibiotics studied) ARG abundance did not appear to be a function of agricultural activity or degree of wastewater impact. Therefore, ARG abundance in the studied lakes is likely not influenced by antibiotic usage, but rather may be influenced by the presence of heavy metals that are known to co-select for ARGs.

Project Results Use and Dissemination

This project led to the production of chapters in the PhD dissertations of both Kyle Sandberg and Jill Kerrigan. Manuscripts will be submitted to the journals *Science of the Total Environment* and *Environment Science and Technology Letters*. Copies of manuscripts will be provided upon publication. The results of this work have been presented at least nine times at national and local conferences.



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

Date of Report: August 21, 2017
Date of Next Status Update Report:
Date of Work Plan Approval: June 4, 2014
Project Completion Date: June 30, 2017
Does this submission include an amendment request? Yes

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: \$300,00

Balance: \$0

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:

Sediments from Lake Pepin, Lake Winona, Little Lake Wilson, and Duluth Harbor have been collected. Water and organic content have been determined for all of the sites. Dating for Lake Pepin has been completed and is underway for the other three cores. A suite of antibiotics have been purchased and verification of chromatography and extraction methods is in progress. DNA has been extracted from the Lake Pepin core and is ready for analysis.

Project Status as of July 1, 2015:

DNA has been extracted from sediment samples from all 4 lake cores and 16S rRNA and *int11* qPCR have been performed on all the samples. Water content has been removed from various sediments subsamples via freeze drying. A liquid chromatography tandem mass spectrometry method has been developed that successfully separates the investigated antibiotics. Method development for the sediment extraction is currently in progress. The sediment cores collected during the 2014 summer have been dated using lead-210 methodology and magnetic susceptibility by Dan Engstrom.

Project Status as of January 1, 2016: Surface sediments were collected from the Mississippi and Minnesota Rivers using a dredge. Water and organic content of the sediment was determined and water was removed from river sediment via freeze drying. Protocols for qPCR of 16S rRNA and 22 antibiotic resistance genes have been fully developed. Method development for the quantification of the suite of antibiotics in sediment is ongoing.

Amendment Request (01/12/2016)

Because personnel costs were higher than expected and travel costs lower than expected in Activity 1, \$2,398 is shifted from travel to personnel.

Amendment Approved: 1/14/2016

Project Status as of July 1, 2016: Protocols for 25 antibiotic resistance genes have been fully developed. All 48 genes have been quantified in all of the Minnesota and Mississippi River sediment samples. Twenty four of the genes have been quantified in all of the core sediment samples. A sediment extraction and quantification method for 23 antibiotics has been optimized. Lake Pepin, Lake Winona, and Duluth Harbor sediment cores have been analyzed for the presence of antibiotics.

Project Status as of January 1, 2017: Protocols for 48 genes, including genes conferring resistance to heavy metals and antibiotics, have been developed. All 48 genes have been quantified in all Minnesota and Mississippi River sediment samples and all core samples. The samples from the Little Wilson Lake sediment core and surface sediments samples from Minnesota and Mississippi Rivers have been analyzed for the presence of antibiotics.

Amendment Request (01/18/2017)

As data was collected on gene and antibiotic levels, it was realized that the analyses for genes needed to be re-run to improve detection limits and that the extraction/analytical protocols for tetracyclines needed to be improved. Additionally, other samples for antibiotics needed to be re-extracted and analyzed to improve data quality. This led to over spending on the supplies and instrument analytical time. Additional personnel support was obtained in the form of a student fellowship, so it is requested to move \$5,500 from personnel in Activity 2 and \$22,500 from personnel in Activity to 3 to supplies/instrument time (\$18,000 in Activity 2 and \$10,000 in Activity 3). These shifts provide sufficient funds to cover expenses already incurred and to complete the analyses of antibiotic concentrations before the project completion date.

Amendment Approved: [01/20/2017]

Overall Project Outcomes and Results:

Antibiotics are substances that stop the growth of or kill bacteria. Animal agriculture and human medicine are the largest consumers of antibiotics worldwide. A fraction of the antibiotic administered is excreted in its original form through urine and/or feces. These residues reach aquatic environments through the discharge of wastewater effluent or drainage and surface runoff from agricultural fields to which manure has been applied. The presence of antibiotics in the environment are of concern, because these chemicals may select for and proliferate the occurrence of antibiotic resistance genes (ARGs). ARGs allow bacteria to survive in the presence of an antibiotic. Heavy metals are also known to co-select for ARGs. The World Health Organization has identified antibiotic resistance as one of the major threats to global health. The increase in the prevalence of antibiotic resistant infections, coupled with the decrease in the development of new antibiotics, emphasize the need for new strategies to better understand antibiotic resistance. The goal of the project is to quantify the current and historical levels of selected human and veterinary antibiotic compounds and genes that code for their resistance in lake sediments. Sediment cores collected for three anthropogenically-impacted Minnesota lakes (Lake Pepin, Duluth Harbor, and Lake Winona) and a control lake in Superior National Forest (Little Wilson Lake) were radiometrically dated. The twenty antibiotics included in this study have a mixture of human and/or agricultural uses, some are known natural products, and they span several of the major classifications (sulfonamides, fluoroquinolones, tetracyclines, macrolides). Sediment cores were successful at capturing the usage trends of ten antibiotics. The initial appearance of antibiotics in the sediment core generally agreed with the FDA approval date, which provided further confidence in the dating of the sediment cores and the ability of sediment cores to capture antibiotic usage trends. Ofloxacin, trimethoprim, sulfapyridine, and sulfamethazine were the only antibiotics to be detected in all three anthropogenically-impacted studied lakes with levels up to 91.7, 2.5, 13.1, and 5 ng g⁻¹, respectively. Human-use antibiotics were detected more frequently and at higher concentrations than antibiotics used for veterinary medicine. Also, the degree of antibiotic pollution appeared to be a function of treated wastewater impact. Lake Winona was the most heavily wastewater impacted lake in the study (approximately 63% of the inflow is treated wastewater effluent) and had the highest concentrations and greatest number of antibiotics detected. Treated municipal wastewater is likely the primary contributor to antibiotic pollution in the studied lakes. The abundance of 48 antibiotic, metal, and antibiotic-associated resistance genes were quantified in the sediment cores with detected levels ranging from 10³ to 10⁸ gene copies per gram. Most ARGs included in this study, however, were not consistently quantifiable throughout the sediment cores. Similar concentrations of *bla*_{SHV}, *cadA*, *copA*, *int11*, and *mexB* were measured amongst the sediment cores, but Lake Winona had higher levels of *sul3* and *tet(A)* compared to the other lakes. ARGs levels did not appear to be a function of sediment core depth, and thus the measured levels are at or close to natural, indigenous background levels of the studied genes. Also, (unlike the antibiotics studied) ARG abundance did not appear to be a function of agricultural activity or degree of wastewater impact. Therefore, ARG abundance in

the studied lakes is likely not influenced by antibiotic usage, but rather may be influenced by the presence of heavy metals that are known to co-select for ARGs. This project led to the production of chapters in the PhD dissertations of both Kyle Sandberg and Jill Kerrigan. Manuscripts will be submitted to the journals *Science of the Total Environment* and *Environment Science and Technology Letters*. Copies of manuscripts will be provided upon publication. The results of this work have been presented at least nine times at national and local conferences.

Amendment Request (08/21/2017)

To complete the required analyses, unused travel funds from activity 1 (~\$1,300) were used to conduct the final analyses of antibiotic concentrations of activity 2.

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 in 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: \$ 45,259
Amount Spent: \$ 45,259
Balance: \$ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Core collection	10/30/14	\$ 13,259
2. Core dating and determination of organic content and deposition rates	7/1/15	\$ 32,000

Activity Status as of January 1, 2015: During August and September 2014, sediment samples were collected from Lake Winona, Lake Pepin, Duluth Harbor, and Little Wilson Lake with the assistance and direction of Dr. Engstrom. Cores were extruded vertically immediately after sampling on shore (except for Lake Pepin) at designated intervals, stored in cleaned jars in the dark at -20 °C. The core from Lake Pepin was dated using

magnetic susceptibility at the National Lacustrine Core Facility at the University of Minnesota and was sectioned at the lab after analysis.

Water and organic content have been determined for all of the sediments. As expected, water content is higher in the top intervals. Dating for one core is complete and in progress for the other three cores.

Activity Status as of July 1, 2015: Three sampling trips have been planned throughout July and August 2015 to collect surface sediments from the Minnesota and Mississippi River.

Dan Engstrom has dated the cores from Lake Winona, Little Wilson Lake, and Duluth Harbor using lead-210 methods.

Activity Status as of January 1, 2016: Surface sediments from 12 different locations along the Minnesota and Mississippi Rivers have been collected. DNA has been extracted from each of these samples in triplicate and are waiting molecular analysis using qPCR. Water and organic content of the river surface sediments has been determined via loss-on-ignition tests. Water was removed from representative sediment subsamples via freeze drying; thereafter the samples are stored in the dark at -20°C until further analysis.

Activity Status as of July 1, 2016: Fourteen heavy metals have been quantified in 6 samples from each sediment core using ICP-MS. The same heavy metals have also been quantified in all of the Minnesota and Mississippi River surface sediments.

Activity Status as of January 1, 2017: Activity 1 was completed by July 1, 2016 update. Nothing to report.

Final Report Summary: The four sediment cores collected from Minnesota lakes were dated. Sediment fluxes as a function of time and focusing-factors specific to each sediment core were calculated to assess historical trends on a whole-lake scale. The organic and water content of sediment was measured at intervals of the sediment core. Results from Activity 1 were applied to data gathered from Activity 2 and 3.

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 ¹³C-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 ¹³C₁₂-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: \$ 140,741
Amount Spent: \$ 140,741
Balance: \$ 0

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	\$ 87,241
3. Calculate accumulation rates	1/31/17	\$ 10,000

Activity Status as of January 1, 2015: An initial suite of antibiotics (6 sulfonamides, 2 macrolides, 3 tetracyclines, 2 fluoroquinolones, carabdox, trimethoprim, triclosan, and 4 degradation products) have been selected and purchased for this study. Verification of chromatography and extraction methods is in progress.

Activity Status as of July 1, 2015: A liquid chromatography tandem mass spectrometry method has been developed that successfully separates the targeted antibiotics. This instrument will be used to quantify antibiotic levels in the sediment extracts. Limits of quantification and detection are currently being evaluated. Method development for the extraction process is in progress, and measurements of concentrations in samples will commence as soon as the process is optimized.

Activity Status as of January 1, 2016: Adjustments to the liquid chromatography tandem mass spectrometry method were made due to matrix effects in the sediment extracts. Method development for the extraction of the suite of antibiotics from the sediment is ongoing. Once the method is optimized, we expect to be able to process samples and get back on schedule.

Activity Status as of July 1, 2016: A sediment extraction and quantification method was optimized for 23 antibiotics, including 4 major degradation products. Sediment cores from Duluth Harbor, Lake Winona, and Lake Pepin have been analyzed for the presence of antibiotics. In Lake Winona, historical trends for 5 sulfonamides, 2 fluoroquinolones, and 2 tetracyclines were observed. Lincomycin, trimethoprim, 3 sulfonamides, 1 fluoroquinolone, and 1 tetracycline were all detected in Lake Pepin. Fewer antibiotics (trimethoprim, 1 sulfonamide, and 2 fluoroquinolones) were quantified in Duluth Harbor’s sediment core. Thus far, we have been able to make some preliminary observations. In general, sediment cores provide a historical record for select antibiotics in several Minnesota lakes. In many cases, the presence of the antibiotic occurred around the initiation of mass production/introduction into clinical use, excluding natural production and contamination. The degree of anthropogenic impact had a great effect on the number of antibiotics detected and their concentration.

Activity Status as of January 1, 2017: The historical record of antibiotics was investigated in the control lake, Little Wilson Lake. Analysis shows no anthropogenic source or natural production of antibiotics into the control lake. Antibiotic concentrations were also measured in surface river sediments from the Minnesota and Mississippi River. Our spatial study suggests that select antibiotics travel downstream from agriculture and urban sources. Re-extractions/re-analyses to improve detection limits and reworking of the methods for tetracyclines were necessary and are ongoing.

Final Report Summary: All analyses to quantify levels of antibiotics within the sediment cores have been completed. Two extraction methods were developed to quantify the presence of twenty antibiotics (six

sulfonamides, four tetracyclines, four fluoroquinolones, three macrolides, trimethoprim, lincomycin, and carbadox) in the sediment samples. No antibiotics were detected in the control lake, Little Wilson Lake. A historical record for ten human and/or animal-use antibiotics (four sulfonamides, three fluoroquinolones, one macrolide, trimethoprim, and lincomycin) was faithfully captured in the other sediment cores collected from Duluth Harbor, Lake Pepin, and Lake Winona. Ten other antibiotics were not detected. Ofloxacin, trimethoprim, sulfapyridine, and sulfamethazine were detected in all of the anthropogenically-impacted studied lakes with maximum concentrations reaching 91.7, 2.5, 13.1, and 5 ng g⁻¹, respectively. The initial appearances of antibiotics in the sediment cores were generally near their FDA approval dates, which further validates the dating from Activity 1. Of the antibiotics that were detected, fluoroquinolone concentrations were higher than any other antibiotic classes. Fluoroquinolone levels were up to 70-fold greater in Lake Winona, 14-fold in Lake Pepin, and 8-fold in Duluth Harbor, than the other detected antibiotics.

Antibiotics that are partially or fully used for human chemotherapy were more frequently detected in the sediment cores. Therefore, the dominant source of antibiotic pollution in the studied lakes likely derives from treated municipal wastewater effluent. Levels of antibiotic pollution also appeared to be a function of anthropogenic impact. The highest levels of antibiotics were measured Lake Winona, the most wastewater impacted lake with 63% of the inflow as treated wastewater effluent. Eight of the human-use antibiotics included in this study are on the World Health Organization (WHO) list of essential medications, which is a registry of pharmaceuticals that are needed for a basic human health-care system. Six of the eight antibiotics that were on WHO list were detected in at least one of the lakes. The WHO list may serve as a catalog of frequently used drugs for which the fate and transport in the environment need to be more fully understood. Antibiotics that are known to be naturally produced by bacteria were not detected as frequently as the antibiotics that are not natural products. Therefore, synthetic antibiotics may be less susceptible to degradation in aquatic systems.

ACTIVITY 3: Measurement of antibiotic resistance 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 -20°C 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 (*int11*), 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: \$ 114,000
Amount Spent: \$ 114,000
Balance: \$ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. DNA extraction and purification	5/31/15	\$ 35,000
2. Quantify known antibiotic resistance genes	4/30/17	\$ 69,000
3. Data synthesis, reporting, and recommendations	6/30/17	\$ 10,000

Activity Status as of January 1, 2015: DNA has been extracted from the Lake Pepin core and is ready for analysis.

Activity Status as of July 1, 2015:

DNA has been extracted from sediment samples from all 4 lake cores and 16S rRNA and *int11* qPCR have been performed on all the samples.

Activity Status as of January 1, 2016: DNA has been extracted from all the surface sediments from the Minnesota and Mississippi Rivers collected during the summer of 2015. Protocols for measuring the 16S rRNA gene as well as 22 different antibiotic resistance genes have been developed. Quantification will commence during the next reporting period.

Activity Status as of July 1, 2016: Protocols to quantify 25 more antibiotic resistance genes have been fully developed. Of the 48 total genes, 24 genes have been quantified in all of the sediment core samples. Quantification of the remaining genes will commence during the next reporting period.

Activity Status as of January 1, 2017: Protocols have been developed to quantify a total of 48 genes. All 48 genes have been quantified in the Minnesota and Mississippi River samples as well as the core samples. Additional analyses were performed to improve detection limits.

Final Report Summary: All antibiotic resistance gene (ARG) analyses of the sediment core samples have been completed. Protocols to quantify 48 genes were developed. Concentrations of antibiotic and metal resistance genes ranged from 10^3 to 10^8 gene copies per gram of sediment. Several ARGs were generally not quantifiable in the sediment cores. A few ARGs (*bla_{SHV}*, *cadA*, *copA*, *int11*, and *mexB*) had similar concentrations throughout the sediment cores. Lake Winona had noticeably higher levels of *sul3* and *tet(A)*. This study saw no correlation between the levels of agricultural activity and degree of treated municipal wastewater and concentrations of ARGs in lake sediments. The abundance of ARGs did not appear to a function of sediment core depth, thus the quantities measured are at or close to natural, indigenous background levels of these genes. Alternatively, the presence of heavy metals within the sediments were found to strongly correlate to ARG levels.

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: Nothing to report.

Status as of July 1, 2015: Nothing to report.

Status as of January 1, 2016: Nothing to report.

Status as of July 1, 2016: Two posters and one oral presentation were given at the Environmental Sciences: Water Gordon Research Conference. Two presentations will be given at the American Chemical Society National Meeting. We anticipate journal manuscripts to be ready soon.

Status as of January 1, 2017: An oral and poster presentation on antibiotic accumulation rates in Minnesota lakes was given at the Minnesota Water Resources Conference and Minnesota Conference on the Environment, respectively. Journal manuscripts are in preparation.

Final Report Summary: This work will be disseminated and archived via peer-reviewed publications, reports to LCCMR, and presentations at conferences. Sediment samples have been frozen for potential future research projects and sediment extracts have been archived for potential future analyses. This work produced chapters within Ph.D. dissertations for both Kyle Sandberg and Jill Kerrigan. Manuscripts will be submitted to the journals *Science of the Total Environment* and *Environment Science and Technology Letters*. To date, six oral and poster presentations have been given at national conferences and three presentations at local conferences on this study.

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, microbiological/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 Equivalent (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	\$ 125,000	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	\$ 125,000	

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: August 4, 2017

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Revised Activity 1 Budget 8/4/17	Amount Spent	Activity 1 Balance	Activity 2 Budget	Revised Activity 2 Budget 8/4/17	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM													
Personnel (Wages and Benefits)	\$26,898	\$26,898	\$26,898	\$0	\$107,500	\$107,500	\$107,500	\$0	\$90,000	\$90,000	\$0	\$224,398	\$0
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 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. Quantification 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	\$16,000	\$16,000	\$0								\$16,000	\$0
Equipment/Tools/Supplies													
Supplies including chemical standards, isotope standards, microbiological/DNA extraction kits, instrument/analytical time for antibiotic and DNA analysis, solvents, consumable supplies, notebooks, software licenses	\$1,000	\$1,000	\$1,000	\$0	\$30,000	\$31,241	\$31,241	\$0	\$22,000	\$22,000	\$0	\$54,241	\$0
Maintenance and repair of laboratory equipment required for analyses and experiments					\$2,000	\$2,000	\$2,000	\$0	\$2,000	\$2,000	\$0	\$4,000	\$0
Travel expenses in Minnesota													
Mileage charges and university vehicle rental charges for trips to collect water samples. Hotel/meal charges if overnight stay required.	\$2,602	\$1,361	\$1,361	\$0								\$1,361	\$0
COLUMN TOTAL	\$46,500	\$45,259	\$45,259	\$0	\$139,500	\$140,741	\$140,741	\$0	\$114,000	\$114,000	\$0	\$300,000	\$0