M.L. 2013 Project Abstract
For the Period Ending June 30, 2016

PROJECT TITLE: Antibiotics in Minnesota Waters – Phase II Mississippi River
PROJECT MANAGER: Kristine Wammer
AFFILIATION: University of St. Thomas
MAILING ADDRESS: Department of Chemistry, 2115 Summit Avenue, OSS 402
CITY/STATE/ZIP: St. Paul, MN 55105
PHONE: (651) 962-5574
E-MAIL: khwammer@stthomas.edu
WEBSITE: http://www.stthomas.edu/chemistry/faculty/kristine-h-wammer.html
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2013, Chp. 52, Sec. 2, Sub. 05h

APPROPRIATION AMOUNT: $203,000

Overall Project Outcomes and Results

This project was Phase 2 of a two-part ENRTF-funded study designed to examine the significance of antibiotics and antibiotic resistance in Minnesota surface waters. Both phases of the study analyzed the following:

- Antibiotic concentrations. Cutting-edge analytical techniques were developed to measure antibiotics at concentrations as low as parts per trillion.
- Antibiotic resistance genes. Quantitative polymerase chain reaction (qPCR) was used to quantify several antibiotic resistance genes.
- Antibiotic-resistant bacteria. Culture-based techniques were used to compare ability of bacteria from various sites to grow in the presence of elevated concentrations of antibiotics.

Phase 1, which ended in 2013, focused on a portion of the Minnesota River basin. The results showed that municipal wastewater treatment plants were a significant source of antibiotics, resistance genes, and antibiotic-resistant bacteria; elevated levels of all three were found in waters impacted by wastewater treatment plant effluent. These findings motivated Phase 2, where the focus shifted to surface waters that serve as drinking water sources and tap water samples and therefore a more direct potential connection to human health impacts. Based on the results of Phase 1, we decided to focus primarily on antibiotics used in human rather than agricultural medicine.

Phase 2 initially focused on the Mississippi River, including St. Cloud, Minneapolis, and St. Paul. Discussions with the Drinking Water Protection section of the Minnesota Department of Health about sites potentially impacted by wastewater led us to expand our study to Ely (Burntside Lake), Grand Marais (Lake Superior), Moorhead (Red River) and Burnsville (Kramer quarry). In general, no measurable antibiotic concentrations, no elevated levels of antibiotic-resistant bacteria, and no antibiotic resistance genes were found in drinking water sources. Development of a new membrane filtration technique allowed us to find antibiotic resistance genes in tap water samples at extremely low levels; the importance of these exceptionally low levels with respect to human health is unclear.

Project Results Use and Dissemination

Four St. Thomas undergraduate students have presented this work at American Chemical Society national meetings; two in 2014, one in 2015, and one in 2016. Dwight Stoll (Gustavus Adolphus) presented at the Quality Assurance meeting of Region 6 of the Environmental Protection Agency in Fall 2015. Kris Wammer (St. Thomas) has presented this work at two national meetings; the Fall 2015 Society of Environmental Toxicology and Chemistry meeting in Salt Lake City, and the Fall 2016 ACS meeting in Philadelphia. A manuscript detailing the findings from this work is also currently in preparation. In addition, we have in the past and will continue to engage relevant personnel at the state level, in particular from state agencies such as MDH, through meetings and formal talks. The MN One Health Antibiotic Stewardship Collaborative, which both Tim LaPara and Kris Wammer participate in, will help facilitate continued interactions with Minnesota stakeholders.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2013 Work Plan Final Report

Date of Status Update Report: August 15, 2016

Final Report

Date of Work Plan Approval: June 11, 2013

Project Completion Date: June 30, 2016

PROJECT TITLE: Antibiotics in Minnesota Waters - Phase II Mississippi River

Project Manager: Kristine Wammer

Affiliation: University of St. Thomas

Mailing Address: Department of Chemistry, 2115 Summit Avenue, OSS 402

City/State/Zip Code: St. Paul, MN 55105

Telephone Number: (651) 962-5574

Email Address: khwammer@stthomas.edu

Web Address: http://www.stthomas.edu/chemistry/faculty/kristine-h-wammer.html

Location: Anoka, Clay, Cook, Dakota, Hennepin, Ramsey, Sherburne, Stearns, St. Louis

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$203,000**
	Amount Spent:	**$194,320**
	Balance:	**$8,680**

Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 05h

Appropriation Language:
$203,000 the first year is from the trust fund to the commissioner of natural resources for an agreement with the University of St. Thomas to measure antibiotic concentrations and antibiotic resistance levels and assess the contributions of farm runoff and wastewater treatment in a portion of the Mississippi River. This appropriation is available until June 30, 2016, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Antibiotics in Minnesota Waters: Phase 2, Mississippi River

II. PROJECT STATEMENT:

Pharmaceuticals and personal care products have gained significant attention in recent years as emerging contaminants in the environment, including attention from legislative bodies. The MN legislature passed a bill in 2009 regulating human pharmaceutical disposal, and bills have been introduced in recent sessions of the U.S. Congress that would restrict the use of antibiotics for agricultural purposes due to concerns over harm to human health related to the development of antibiotic resistance. While the environmental occurrence of these compounds has spurred interest, major gaps still remain in our understanding of their significance and potential health and ecological impacts. The critical question of which, if any, emerging contaminants are of the most direct concern to human health is still largely unanswered. Because the threat of decreased efficacy of antibiotics due to increases in antibiotic resistance levels is such a significant human health threat, this class of pharmaceuticals is a priority for further study.

The goal of this project is to study the development of antibiotic resistance due to the presence of antibiotics and antibiotic resistance genes in farm runoff and in wastewater treatment plant effluents, which then subsequently impact surface waters. We will study a portion of the Mississippi River from north of St. Cloud to south of Kellogg. This project team is currently working on a similar ENRTF-funded study in a portion of the Minnesota River. Our findings to date suggest that wastewater treatment plant effluents are a potentially important path for both antibiotics and antibiotic resistance genes to reach surface waters. This is consistent with findings by a recent USGS study that reported elevated levels of pharmaceuticals, including one antibiotic (sulfamethoxazole) in wastewater treatment plant effluents throughout Minnesota. We now propose to address the pressing question of whether drinking water is being impacted, and whether this is observed on a larger scale.

Samples will be obtained from locations selected to allow comparison of primarily agricultural (including drainage ditches), primarily residential/industrial (including wastewater treatment effluents), and mixed inputs to the Mississippi River. In addition, we plan to collect samples near drinking water intakes and tap water from Minneapolis, St. Paul, and St. Cloud. The project will assess current antibiotic concentrations and antibiotic resistance levels for members of four major classes of antibiotics used in both human medicine and agriculture: tetracyclines, sulfonamides, macrolides, and fluoroquinolones. A unique strength of this project is that the project team combines expertise in cutting-edge analytical chemistry techniques with expertise in rigorous microbiology and molecular biology techniques to characterize each water sample. We intend to attempt to complete the bulk of the project work within the first two years; a project duration of three years is specified in case weather or other factors require more time for successful completion of the project.

III. PROJECT STATUS UPDATES:

Project Status as of January 2014:

We have thus far selected eight sample sites of interest and five target antibiotics to study. We have completed three preliminary sampling trips and done method development work. Quantification of antibiotic resistant organisms has been successfully performed on river samples, but method development continues to successfully culture quantifiable numbers of bacteria from tap water samples. DNA has been extracted from all samples collected, and qPCR experiments to quantify antibiotic resistance genes from these samples will be performed within the next several months. Method development is also ongoing for detecting the very low concentrations of antibiotics likely to be present in drinking water sources.

A note of explanation: on advice from LCCMR staff, the word “summer” was deleted from the budget in describing the salary allocated for PI Wammer. Part of that salary will be used for effort during the academic year, instead of completely during the summer months.

Project Status as of October 2014:

Method development has improved, and we are now able to quantify cultivable bacteria and target genes from all sources of interest, including tap water. Detection limits for antibiotics are approaching the single

digit parts per trillion level. Although we took two sampling trips this past summer, with another scheduled in early November, we will conduct several additional trips next spring and summer to take advantage of current methodology. In the coming months, remaining qPCR experiments will be performed on previously obtained DNA samples.

Amendment Request (10/31/14): When we proposed the original budget, we failed to realize that in some cases, depending on sample scheduling and location, it would be much more cost-effective and time-efficient to send samples to Gustavus for measurements of antibiotic concentrations rather than either delivering the samples or having a Gustavus student present for the sampling. Therefore, we would like to add shipping costs as an allowable expense; we have modified the Equipment/Tools/Supplies budget line to include Shipping. **Approved by the LCCMR November 14, 2014.**

Project Status as of March 2015: We conducted one sampling trip in the fall. We have completed qPCR analysis on all samples collected during the last sampling season, and see variability among sites but no consistent trends. We continue to work on method development in preparation for several anticipated sampling events beginning later this spring. Automated, online extraction should allow for improved quantitation of antibiotic concentrations. We also anticipate collecting significantly larger volumes of water using a dead-end membrane filtration approach to improve detection limits for antibiotic resistance genes in drinking water, and intend to add some new sites beyond those we have examined to date.

Amendment Request (3/27/15): We intend to complete the work over a three-year period rather than the originally proposed two-year period. We still have a lot of sampling we wish to do, taking advantage of methods we have developed over the course of the project thus far, and will need to do the resulting analysis as well. **Amendment request approved by the LCCMR 3-31-15.**

Project Status as of October 2015: Over the past several months we made significant progress. We conducted four trips to sample our regular Mississippi River sites and added samples from several new additional surface drinking water source sites around the state. We also successfully implemented a new membrane filtration approach that has allowed us to obtain measurable quantities of antibiotic resistance genes in tap water for the first time. All work to date has suggested that there are no consistent elevated levels of antibiotic resistance or measurable antibiotic concentrations in any drinking water sources sampled. Work to quantify the antibiotic resistance genes in various tap water samples is ongoing.

Overall Project Outcomes and Results: This project was Phase 2 of a two-part ENRTF-funded study designed to examine the significance of antibiotics and antibiotic resistance in Minnesota surface waters. Both phases of the study analyzed the following:

- Antibiotic concentrations. Cutting-edge analytical techniques were developed to measure antibiotics at concentrations as low as parts per trillion.
- Antibiotic resistance genes. Quantitative polymerase chain reaction (qPCR) was used to quantify several antibiotic resistance genes.
- Antibiotic-resistant bacteria. Culture-based techniques were used to compare ability of bacteria from various sites to grow in the presence of elevated concentrations of antibiotics.

Phase 1, which ended in 2013, focused on a portion of the Minnesota River basin. The results showed that municipal wastewater treatment plants were a significant source of antibiotics, resistance genes, and antibiotic-resistant bacteria; elevated levels of all three were found in waters impacted by wastewater treatment plant effluent. These findings motivated Phase 2, where the focus shifted to surface waters that serve as drinking water sources and tap water samples and therefore a more direct potential connection to human health impacts. Based on the results of Phase 1, we decided to focus primarily on antibiotics used in human rather than agricultural medicine.

Phase 2 initially focused on the Mississippi River, including St. Cloud, Minneapolis, and St. Paul. Discussions with the Drinking Water Protection section of the Minnesota Department of Health about sites

potentially impacted by wastewater led us to expand our study to Ely (Burntside Lake), Grand Marais (Lake Superior), Moorhead (Red River) and Burnsville (Kramer quarry). In general, no measurable antibiotic concentrations, no elevated levels of antibiotic-resistant bacteria, and no antibiotic resistance genes were found in drinking water sources. Development of a new membrane filtration technique allowed us to find antibiotic resistance genes in tap water samples at extremely low levels; the importance of these exceptionally low levels with respect to human health is unclear.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collect samples and quantify cultivable antibiotic resistant organisms at targeted Mississippi River sites

Description:

Enumerating "antibiotic resistance" poses a unique challenge because of the diversity of microorganisms in nature and the diversity of antibiotics studied. Therefore, we will use two techniques that provide complementary data to give us the most accurate information: quantitative polymerase chain reaction (qPCR, described in Activity 3), as well as the cultivation-based approaches of Activity 1. The qPCR technique allows us to quantify specific genes that encode antibiotic resistance, but the organisms that harbor the genes (and their characteristics) remain unknown. The benefit of the cultivation-based approach is that it provides bacterial isolates that can be analyzed further (for example, we will determine their resistance to multiple antibiotics). Samples will be obtained from locations selected to allow comparison of primarily agricultural, primarily residential/industrial, and mixed inputs to the Mississippi River. In addition, we will collect samples from areas near drinking water intakes and tap water samples. Samples will be collected from each site on several occasions, varying seasonally and with rainfall events as much as possible. Bacteria from these samples will be cultivated on two different types of solid growth media amended with a range of concentrations of each of four antibiotics: tetracycline, sulfamethoxazole (a sulfonamide), tylosin (a macrolide), and ciprofloxacin (a fluoroquinolone). Cultivable antibiotic-resistant bacteria will be enumerated and compared to the number of bacteria able to grow on non-amended growth media. Resistant bacteria will also be isolated and tested for resistance to other classes of antibiotics.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 59,943
Amount Spent: $ 56,531
Balance: $ 3,412

Activity Completion Date:

Outcome	Completion Date	Budget
1. Develop any new necessary methods based on findings from the first several sampling events.	May 2014	$ 8,991
2. Collect samples from various sites, varying seasonally and with rainfall events.	November 2015	$ 14,986
3. Enumerate antibiotic-resistant bacteria for samples collected at each sampling event by cultivating bacteria on growth media amended with a range of concentrations of the 4 target antibiotics: tetracycline, tylosin (a macrolide), sulfamethoxazole (a sulfonamide), and ciprofloxacin (a fluoroquinolone) in addition to any new antibiotics identified as targets during the project. Isolate resistant bacteria and test their resistance to other classes of antibiotics.	June 2016	$ 35,966

Activity Status as of January 2014: In the late summer and fall, we completed three Mississippi River sampling trips (July 29-30, August 21, and September 28). Samples were collected upstream of and near drinking water intakes. In addition, tap water samples were collected in St. Cloud and St. Paul. On the first two trips we

collected samples both from the St. Cloud area and the Twin Cities; the third trip focused on the Twin Cities only. Results from Phase 1 of this project, which has focused on a portion of the Minnesota River, informed the design of this study. Our work to date has shown that wastewater treatment effluents appear to be more significant sources of antibiotics and antibiotic resistance genes than agricultural runoff. Therefore, we are focusing here primarily on human antibiotics. We have chosen to study five antibiotics heavily used in human medicine: amoxicillin, azithromycin, ciprofloxacin, sulfamethoxazole, and trimethoprim. The primary focus for these initial sampling trips has been method development. We are working to determine the correct antibiotic concentrations and water sample dilutions to allow us to quantify cultivable organisms on antibiotic-amended solid media. Our biggest methodological challenge will be to culture bacteria from tap water samples. We are working with large volumes of filtered tap water, and new types of growth media, to address this challenge prior to starting sampling in earnest next spring.

Activity Status as of October 2014: We have made significant strides in our method development since our last report. All five antibiotics mentioned in the previous status report are still being studied. Methods are now reliable for all but ampicillin; optimal concentrations for solid media plates are still being determined for this drug. We are also now able to obtain countable bacteria from tap water samples. Although we will make one more sampling trip yet this fall, primarily in support of Activities 2 and 3, we plan to conduct several trips next spring and summer to collect quantifiable data for enumerating antibiotic-resistant bacteria including in tap water samples. This past summer, we made two sampling trips, on June 16 and July 7. In both cases, antibiotic-amended solid media (both high and low nutrient media) was used to compare bacteria from all sites other than drinking water samples. With the exception of the low nutrient media samples from the June 16 trip, and ampicillin samples in general, samples were quantifiable. Although small differences are observed among the sites, for the most part no consistent trends in these differences have yet been observed.

Activity Status as of March 2015: One additional sampling trip was conducted in November 2014. Culturing was not done for these samples; these were used only for measuring antibiotic concentrations for Activity 2 and the qPCR experiments associated with Activity 3. Sampling efforts related to this task will resume in late spring. We plan to add some new drinking water source sampling sites for our upcoming sampling trips; these were determined with input from MDH through discussions in January 2015. We also hope to use a novel approach to collect samples using a dead-end membrane filtration approach. This new method should allow us to collect and concentrate much larger sample volumes (increasing from 10 L to 1000 L or more), which should help us improve our detection limits substantially.

Activity Status as of October 2015: Over the course of the past several months, we did extensive sampling. Four trips were taken to collect samples from our usual Mississippi River sample sites (St. Cloud and Twin Cities areas), on June 8, June 29, July 20, and August 3. On these trips we collected water to measure cultivable bacteria for Activity 1, to measure antibiotic concentrations for Activity 2, and to get antibiotic resistance genes for quantification as part of Activity 3. Because we have never seen elevated resistance levels at any of the Mississippi River sites, we also decided to add several new surface water drinking water source sites for one-time (or sometimes more) sampling, especially targeting sites that may be anticipated to be impacted by wastewater or other contamination. We selected these sites in consultation with the Drinking Water Protection section at the Minnesota Department of Health. Water from the Kraemer quarry in Burnsville was sampled on June 18 and July 13 and a drinking water intake in Moorhead (source is the Red River) was sampled on June 22. Samples were also collected from intakes in Ely (source is Burntside Lake), Grand Marais (source is Lake Superior), and Grand Marais treated wastewater effluent (which is discharged less than one mile upstream of the drinking water intake) on July 9-10. In addition to source water, finished tap water was collected from several locations using the new dead-end membrane filtration technique, which allowed for gene analysis in very large volume samples (~1000 L to ~10,000 L). In addition to several tap water samples collected at our labs in Minneapolis and St. Paul, samples were collected in Moorhead on June 22, August 18, and September 7, in St. Cloud on July 20 and September 8, and in Ely and Grand Marais July 9-10. Even with all the new sites, we still see

no consistent trends of elevated resistance levels among cultivable bacteria in any drinking source water sampled.

Final Report Summary: Over the course of the project, we focused on five antibiotics: ampicillin, azithromycin, ciprofloxacin, sulfamethoxazole, and trimethoprim. After several initial sampling trips undertaken during the course of method development in 2013, we completed six comprehensive sampling trips. Two took place in the summer of 2014, and the other four took place in the summer/fall of 2015. Based on input from colleagues at the MDH Drinking Water Protection section, we expanded beyond our originally proposed sampling sites to test other locations that might plausibly be impacted by antibiotics. We observed some significant differences in growth of bacteria from various sites on the two types of media and in the presence of the various antibiotics. In general, however, no strong, systematic differences were observed among any of the drinking water source sites studied, suggesting no reason for concern due to elevated levels of antibiotic-resistant organisms at any of the sites studied. Our new dead-end membrane filtration technique, developed as of 2015, has been applied over the past several months to study cultivable bacteria from finished drinking water samples, including on a new media type (R2A) designed for isolating bacteria from potable water. Although bacteria have been grown in the presence of antibiotics from Minneapolis and St. Paul tap water samples, no significant trends have been observed here either. The scope of this project expanded from our original proposal, yet we were able to come in under budget. The unspent funds for this activity were primarily undergraduate student wages. Students successfully applied for internal St. Thomas research grants on several occasions that took the place of these funds.

ACTIVITY 2: Measure antibiotic concentrations at same Mississippi River sites

Description:

We will analyze water samples for the presence of selected antibiotics using methods based on two-dimensional high performance liquid chromatography developed in the laboratory of Dwight Stoll (one of the project partners). These methods have exceptional separation power that will allow us to accurately detect antibiotics even in complicated sample matrices such as those being considered in this work. In the work currently funded by the ENRTF, the Stoll group has successfully measured the concentration of several antibiotics in drainage ditches, the Minnesota River, and wastewater treatment plant effluents with detection limits in the parts per trillion range. An important aspect of the current work has been the development and implementation of online-Solid Phase Extraction (online-SPE) to reduce carryover, improve analyte recovery, and increase sample throughput. In Phase 2 of this project, we will continue development of our online-SPE approach coupled to two-dimensional HPLC with MS detection, with a focus on improving the sensitivity of the approach by reducing the dimensions of the analytical separation system. We anticipate that these improved levels of sensitivity will be required for work with tap water where the target compounds are unlikely to be present above the high parts-per-quadrillion or low parts-per-trillion range.

Summary Budget Information for Activity 2:

ENRTF Budget: $ 81,841
Amount Spent: $ 80,881
Balance: $ 960

Activity Completion Date:

Outcome	Completion Date	Budget
1. Screen samples collected throughout the first summer of the project for the presence of the 4 target antibiotics.	November 2013	$ 20,460
2. Optimize our methods for the samples of interest. Identify potential new target antibiotics based on initial results, and develop detection methods.	March 2014	$ 27,008
3. Quantify concentrations of the 4 target antibiotics plus any new target antibiotics for samples collected at each sampling event	June 2016	$ 34,373

beginning in Spring 2014.		

Activity Status as of January 2014: In this first portion of the grant period we have focused on analytical method development, with an eye toward the improvements in methodology and detection limits that will be required to reliably quantify the target antibiotics at the low parts-per-trillion level. Specifically, we have been working on instrument hardware and software that will enable parallel selective two-dimensional separations focused on the compounds of interest. This has been a highly collaborative effort with Agilent Technologies. Most recently we have been evaluating the robustness of a two-dimensional liquid chromatograph with prototype hardware and software components in play. We expect that we will begin intensive work with water samples on this system in the first quarter of 2014.

Activity Status as of October 2014: Since January 2014 we have analyzed approximately 100 water samples by online-SPE coupled with two-dimensional HPLC and TOF-MS detection. This includes both samples provided by the Wammer Group from the St. Paul area sampling sites, as well as other samples obtained locally for method development purposes. To date we have developed a reliable method for analyzing 5 mL of water per analysis, but this volume is too small to obtain the detection limits desired (sub-ppt) for analysis of drinking water. Thus, our current effort is focused on extending the sample volume limit to 50 mL. This will involve prototype instrument components from our close collaborator Agilent Technologies. We will continue this development effort through the winter, and implement the resulting method for further analysis of samples from the Wammer group in the spring of 2015.

Activity Status as of March 2015: In the most recent quarter our method development has been focused on developing instrument hardware and a control scheme to enable automated, online extraction of 50-mL water samples in a robust and reliable way. At this point we have demonstrated robust operation for samples up to 25 mL, and shown that quantitation is predictable and reliable in the range of sample volumes between 1 and 25 mL. This spring we will continue pushing toward 50-mL extractions, and finally implement this extraction methodology in our 2D-LC-MS system for analysis of water samples collected later this spring.

Activity Status as of October 2015: Over the summer our work was focused on two aims: 1) pushing the online-SPE methodology toward automated extraction of 50-mL samples; and 2) applying the online-SPE-2D-LC-MS methodology in the analysis of water samples collected by the Wammer group during this period. While we have demonstrated automated online-SPE for 50-mL samples, we now know that at this scale the approach works well only for the least hydrophilic of our target analytes. It has become clear that realizing these large extraction volumes in practice for more hydrophilic target compounds will require the development of new and/or possibility mixed chemistry extraction materials that are more effective at retaining hydrophilic compounds. The analyses of the large majority of samples collected by the Wammer group yielded no detectable concentrations of the target antibiotics. Nevertheless, in the process of working with these samples we did demonstrate the utility of the high resolving power of 2D-LC in reducing matrix effects on ionization during MS detection of the target compounds.

Final Report Summary: In the course of this project we developed a robust approach to analyzing samples of up to 50 mL by online-SPE-2D-LC-MS. Compared to conventional methods, this approach minimizes sample handling, which in turn minimizes analyte losses and opportunities for sample contamination. With this approach we demonstrated detection limits in the range of 10 parts-per-trillion for several antibiotics, even while using a time-of-flight mass spectrometer. It is expected that coupling our 2D-LC-MS approach to a triple quadrupole mass spectrometer would yield detection limits in the sub-parts-per-trillion range. We also demonstrated that the second dimension of chromatographic separation prior to mass spectrometric detection was helpful for reducing the effects of the sample matrix on suppressing ionization during the detection step. This has the benefit of improving detection limits and the reliability of the overall analytical method. With these detection limits, most of the analyses of drinking water source or tap water samples did not yield any detectable concentrations of antibiotics. An important finding from this work is that materials suitable for use as trapping

cartridges in the online-SPE approach, particularly for hydrophilic molecules, are very limited. As an outcome of this work we have engaged a major manufacturer of chromatographic materials to address this gap, which will ultimately improve the applicability of online-SPE for analysis of hydrophilic molecules in the environment. For the purposes of this project, the finding of no measurable quantities of antibiotics in surface water or tap water samples, even with a technique allowing us to have such low detection limits, allows us to report that the presence of antibiotics is very likely not of concern for the drinking water sources studied here.

ACTIVITY 3: Quantify antibiotic resistance genes

Description:

Quantitative polymerase chain reaction (qPCR) will be used to provide complementary information to that obtained by the cultivation-based approaches of Activity 1. The qPCR technique involves concentrating the bacteria within the samples on filters and then extracting/purifying the DNA of any gene of interest. We will target genes that confer resistance to the antibiotics of the tetracycline (*tet*(A), *tet*(X), and *tet*(W)), sulfonamides (*sul1*), macrolide (*ermB*), and fluoroquinolone classes (*qnrA*). In addition, we will target Class 1 integrons (*intI1*), which are associated with multiple antibiotic resistance.

Summary Budget Information for Activity 3:

ENRTF Budget: $ 61,216
Amount Spent: $ 56,907
Balance: $ 4,309

Activity Completion Date:

Outcome	Completion Date	Budget
1. Isolate DNA from samples collected at each sampling event.	November 2015	$ 45,912
2. Quantify genes conferring resistance to the 4 original classes of antibiotics, in addition to Class 1 integrons, plus any new classes of interest for samples collected throughout the year.	June 2016	$ 15,304

Activity Status as of January 2014: We have extracted DNA from all of the samples collected to date. Because we have not worked with tap water samples previously, we need to perform some preliminary experiments to determine the optimal amount of water to run through each filter, and the number of filters to combine to get measurable gene quantities by qPCR. Once these preliminary experiments are completed, we will begin performing qPCR experiments on all of the extracted DNA samples.

Activity Status as of October 2014: DNA has been extracted from all samples obtained on both trips this past summer. In addition, qPCR experiments targeting qnrA and intI1 have been completed. Total numbers of bacteria, as measured by 16S rRNA, are fairly consistent from site to site with the exception of tap water samples, which are much lower. No measurable quantities of intI1 or qnrA have been detected in the tap water samples. There are significant differences in quantities of intI1 and qnrA among source water sites, but the trends are not yet consistent enough to confidently report; further analysis is needed over the coming months.

Activity Status as of March 2015: All qPCR experiments have been completed for samples from last year. Although there is site-to-site variability, there are still no consistent trends to report among the various sites. The quantity of DNA extracted from drinking water is high enough to quantify 16S rRNA genes but not antibiotic resistance genes, so hopefully the new dead-end membrane filtration method (described under Activity 1) will produce higher quantities.

Activity Status as of October 2015: All qPCR experiments have been completed for source water samples from this year. As seen previously, although there is site-to-site variability, no consistent trends are observed among the various sites. The new dead-end membrane filtration method has significantly improved our ability to detect genes in tap water. Based on this new technique, we are now able to quantify antibiotic resistance genes in the

tap water samples. Work on the qPCR experiments associated with the fourteen tap water samples collected through membrane filtration is ongoing.

Final Report Summary: All qPCR experiments have been completed, including fourteen tap water samples processed using the dead-end membrane filtration technique. After our initial work targeting a relatively small number of antibiotic resistance genes, we also extended this work by quantifying 47 antibiotic resistance genes using a novel microfluidic method. As previously observed, although there is site-to-site variability, no consistent trends are observed among the various sites. Interestingly, however, quantifiable amounts of each of the antibiotic resistance genes were found in tap water samples. This means that although there are not elevated levels of antibiotic-resistant organisms, antibiotic resistance genes, or antibiotics at drinking water source sites, measurable quantities of the antibiotic resistance genes that are present are making it through the drinking water treatment process and into finished drinking water. It is important to note that the quantity of these antibiotic resistance genes is extremely low and that these genes were only detectable because of the application of the novel, dead-end membrane filtration method that enabled collection of exceptionally large sample volumes (> 5,000 liters) compared to conventional methods (< 1 liter). Unused budget in this activity is, as in Activity 1, primarily due to success in obtaining internal grants for undergraduate student wages.

V. DISSEMINATION:

Description: The results of this study will be disseminated through oral and poster presentations by the students and faculty involved in the project, briefings to the LCCCMR as requested, and peer-reviewed publication. We also intend to present progress on this project periodically to relevant personnel who have been made aware of this project and may be interested in the results, specifically at the Minnesota Department of Health (Drinking Water Protection Section) and the Minnesota Pollution Control Agency.

Status as of January 2014: Maia Moffatt and Jackie Kapla, both undergraduate students at the University of St. Thomas, have submitted abstracts to present work on this project at the spring American Chemical Society (ACS) meeting in March 2014.

Status as of October 2014: Jackie Kapla and Maia Moffatt, both from the University of St. Thomas, presented posters at the Spring 2014 American Chemical Society (ACS) meeting in Dallas, TX. Jackie's poster was "Quantifying Cultivable Antibiotic-Resistant Bacteria in Surface Waters," and Maia's was "Quantifying Bacterial Resistance to Antbiotics in Minnesota Surface Waters." Kris Wammer presented a poster at the 2014 Gordon Research Conference on Environmental Sciences: Water, titled "Antibiotics and Antibiotic Resistance in Surface Waters Impacted by Agricultural and Municipal Inputs." Liz Beck will submit an abstract to present work at the Spring 2015 National ACS meeting in March 2015.

Status as of March 2015: Kris Wammer gave a presentation to and had follow-up discussion with ten Minnesota Department of Health (MDH) and two Minnesota Pollution Control Agency (MPCA) scientists and engineers in November 2014. The two main purposes of this interaction were to disseminate results from Phase 1 and Phase 2 of this project to date and to seek input for what potential additions we might make as we head into the next sampling season in Spring/Summer 2015. Liz Beck (University of St. Thomas student) presented a poster in March at the Spring 2015 ACS National meeting, titled "Integrons and Multiple-Antibiotic Resistant Bacteria in Minnesota Surface Waters."

Status as of October 2015: Kris Wammer gave a presentation describing this work in an invited seminar for the University of Wisconsin Environmental Engineering and Environmental Chemistry programs in October 2015. Kris Wammer also gave a poster describing this work, titled "Antibiotics and Antibiotic Resistance in Minnesota Surface Waters" at the SETAC North America Annual meeting in Salt Lake City in November 2015. Dwight Stoll gave a presentation on two-dimensional liquid chromatography (2D-LC) at the Quality Assurance meeting of

Region 6 of the Environmental Protection Agency in Dallas, Texas, in October of 2015. During this talk he described the use of 2D-LC for environmental analysis, which has been informed by the current project.

Final Report Summary: Since the last status update, Kris Wammer presented this work in November 2015 as part of the Clean Water Lunchtime Brownbag series at the Minnesota Department of Health; her talk was streamed online so people from other parts of the state could attend virtually in addition to the in-person audience. Michael Andreone (University of St. Thomas student) also presented a poster titled "Antibiotic Resistance in Surface Drinking Water Sources and Finished Tap Water" at the Spring 2016 National ACS meeting and Kris Wammer gave an oral presentation at the Fall 2016 National ACS meeting.

To summarize dissemination to date and planned future dissemination to the professional community: four St. Thomas undergraduate students have presented this work at national meetings; Dwight Stoll (Gustavus Adolphus) presented at one professional meeting; Kris Wammer (St. Thomas) has presented this work at two national meetings (SETAC and ACS). A manuscript detailing the findings from this work is also currently in preparation. In addition, we have in the past and will continue to engage relevant personnel at the state level, in particular from state agencies such as MDH, through meetings and formal talks. For example, Kris Wammer will give a talk at the Minnesota Section American Water Works Association (AWWA) conference in September. Kris Wammer and Tim LaPara are both participants in the MN One Health Antibiotic Stewardship Collaborative (http://www.health.state.mn.us/onehealthabx/index.html) and data from this study will almost certainly inform the work of that group.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget:

Budget Category	$ Amount	Explanation
Personnel:	$ 55,963	$23,211 for principal investigator (Wammer), which includes 1.5 months of salary per year plus associated fringe benefits. $32,752 for undergraduate students: two working full-time each summer and three working 6 hours per week during the academic year.
Professional/Technical/Service Contracts:	$ 126,681	$44,840 to University of Minnesota (LaPara) includes 4 weeks of salary per year plus associated fringe benefits ($31,340), lab supplies ($12,500), and travel ($1,000). $81,841 to Gustavus Adolphus College (Stoll) includes 1 month of salary per year plus associated fringe benefits ($14,319), a research technician working 14 hours per week ($34,540), one student working full-time each summer ($12,512), one student working 8 hours per week during the academic year ($5,470), lab supplies ($6,000), instrument access ($8,000), and travel ($1,000)
Equipment/Tools/Supplies/Shipping:	$ 16,356	General lab supplies (e.g. HPLC consumables, antibiotics, nutrient media, petri dishes) and costs for shipping samples to collaborators
Travel Expenses in MN:	$4,000	Mileage reimbursement and meals for approximately 20 sampling trips based on the plan of the Commissioner of Management of Budget.
TOTAL ENRTF BUDGET:	**$ 203,000**	

Explanation of Use of Classified Staff: Salary is included for the project manager (Wammer) and project partners (LaPara and Stoll) who are all on 9-month academic contracts

Explanation of Capital Expenditures Greater Than $3,500: N/A

Number of Full-time Equivalent (FTE) funded with this ENRTF appropriation: 1.7 FTE

Number of Full-time Equivalent (FTE) estimated to be funded through contracts with this ENRTF appropriation: 1.7 FTE

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
University of St. Thomas	$ 10,541	$ 10,541	Salary and fringe benefits for one undergraduate student each summer.
State			
N/A	$	$	
TOTAL OTHER FUNDS:	**$ 10,541**	**$ 10,541**	

VII. PROJECT STRATEGY:

A. Project Partners:

Kristine Wammer, University of St. Thomas, Department of Chemistry ($76,319)

Timothy LaPara, University of Minnesota, Department of Civil Engineering ($44,840)

Dwight Stoll, Gustavus Adolphus College, Department of Chemistry ($81,841)

B. Project Impact and Long-term Strategy:

This project will help us understand the significance of an important class of emerging contaminants, antibiotics, as a potential threat in natural waters. In the first phase of this project, we focused on a selected portion of the Minnesota River that allowed us to investigate the relative importance of agricultural vs. municipal inputs. This work is still ongoing, but results to date suggest that wastewater treatment plants may be more significant sources of both antibiotics and antibiotic resistance genes than agricultural runoff. These results will hopefully be useful for informing future regulations related to wastewater, drinking water, and agriculture. A limitation of the first phase of the project, however, was that the selected portion Minnesota River is not used as a drinking water source. Phase 2 of the project will allow us to determine whether our findings from the Minnesota River are mirrored in a portion of the Upper Mississippi river, and will allow us to measure whether drinking water sources are impacted. This will allow us to more directly study the relevance of this issue as a human health concern.

C. Spending History:

Funding Source	M.L. 2007 or FY08	M.L. 2008 or FY09	M.L. 2009 or FY10	M.L. 2010 or FY11	M.L. 2011 or FY12-13
ENRTF					$190,000 Sub. 5(e)

VIII. ACQUISITION/RESTORATION LIST: N/A

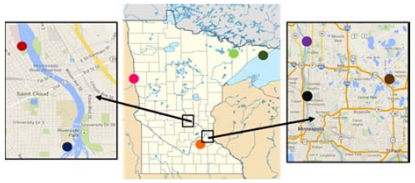
IX. MAP(S): N/A

X. RESEARCH ADDENDUM: Peer reviewed in Phase 1 of project (Assessment of Minnesota River Antibiotic Concentrations, M.L. 2011)

XI. REPORTING REQUIREMENTS:

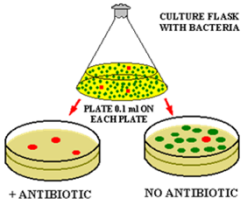
Periodic work plan status update reports will be submitted not later than January 2014, October 2014, March 2015, October 2015, and April 2016. A final report and associated products will be submitted between June 30 and August 15, 2016 as requested by the LCCMR.

Antibiotics in Minnesota Waters – Phase II Mississippi River



SAMPLING

Culture-Based



No elevated levels of antibiotic-resistant organisms found in drinking water sources

Gene-Based

No elevated levels of antibiotic resistance genes found in source waters, but measurable quantities found in tap water

Antibiotics

No elevated levels of antibiotics found in drinking water sources or tap water

Attachment A: Budget Detail for M.L. 2013 Environment and Natural Resources Trust Fund Projects

Project Title: Antibiotics in Minnesota Waters: Phase 2, Mississippi River
Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 05h
Project Manager: Kristine Wammer
M.L. 2013 ENRTF Appropriation: $ 203,000
Project Length and Completion Date: 3 years; June 30, 2016
Date of Update: October 1, 2015

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Balance	Activity 2 Budget	Amount Spent	Balance	Activity 3 Budget	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	**Collect samples and quantify cultivable antibiotic resistant organisms at targeted Mississippi River sites**			**Measure antibiotic concentrations at same Mississippi River sites**			**Quantify antibiotic resistance genes**				
Personnel (Wages and Benefits)	$39,587	$37,048.64	$2,538.36				$16,376	$12,105.58	$4,270.42	$55,963.00	$6,808.78
Kristine Wammer, Project Manager: $23,211 (92% salary, 8% benefits), 1.5 months effort.											
Undergraduate Research Assistants: $32,752 (95% salary, 4% benefits), 2 students full-time for 12 weeks each summer, 3 students working 6 hours per week for 32 weeks during the academic year.											
Professional/Technical/Service Contracts											
University of Minnesota: Timothy LaPara, Principal Investigator. 4 weeks of salary per year plus associated fringe benefits (34.9%). Duties: Responsible for some tests of antibiotic resistance levels including supervision of St. Thomas undergraduate students (Activity 2) ($31,340). General lab supplies (e.g. PCR primers, reagents ($12,500). Travel funds for a few targeted sampling trips independent of the main sampling events ($1000)							$44,840.00	$44,801.78	$38.22	$44,840.00	$38.22
Gustavus Adolphus College: Dwight Stoll, Principal Investigator. 1 month of salary per year plus associated fringe benefits (16.48%). Duties: Responsible for analysis of concentrations of antibiotics and supervision of Gustavus undergraduate students and reseach technician (Activity 1) ($14,319). Research technician 15 hours per week at $20 per hour plus associated fringe benefits (9.48%) ($34,540). 1 student during each summer, 40 hours per week for 12 weeks each year, $10.50 per hour, plus associated fringe benefits and summer housing ($12,512). 1 student during the academic year, 8 hours per week for 32 weeks, $10.50 per hour ($5,470). General lab supplies, e.g. solvents, vials, analytical standards ($6000). LC/MS instrument access				$81,841.00	$80,881.44	$959.56				$81,841.00	$959.56
Equipment/Tools/Supplies/Shipping General lab supplies (e.g. antibiotics, nutrient media, petri dishes, solvents) and costs for shipping samples to collaborators	$16,356.00	$16,355.81	$0.19							$16,356.00	$0.19
Travel expenses in Minnesota Approximately 20 sampling trips, 300 miles round trip, based on the plan of the Commissioner of Management of Budget.	$4,000.00	$3,126.48	$873.52							$4,000.00	$873.52
COLUMN TOTAL	**$59,943**	**$56,531**	**$3,412**	**$81,841**	**$80,881**	**$960**	**$61,216**	**$56,907**	**$4,309**	**$203,000**	**$8,680**