M.L. 2011 Project Abstract
For the Period Ending June 30, 2014

PROJECT TITLE: Assessment of Minnesota River Antibiotic Concentrations
PROJECT MANAGER: Kristine Wammer
AFFILIATION: University of St. Thomas
MAILING ADDRESS: 2115 Summit Ave, OSS 402
CITY/STATE/ZIP: St. Paul, MN 55105
PHONE: (651) 962-5574
E-MAIL: khwammer@stthomas.edu
WEBSITE: http://www.sthomas.edu/chemistry/faculty/wammer.htm
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2011, First Special Session, Chp. 2, Art. 3, Sec. 2, Subd. 05e

APPROPRIATION AMOUNT: $190,000

Overall Project Outcome and Results

While the presence of antibiotics in surface waters has received attention due to concerns about health or ecological impacts, major gaps still remain in our understanding of the scope and significance of this potential problem. The goal of this study was to address the question of whether human or agricultural sources of antibiotics and antibiotic resistant bacteria may be the most significant in surface waters impacted by both. We focused on drainage ditches that receive farm runoff and municipal wastewater treatment plant effluents as possible sources for a portion of the Minnesota River in Southern Minnesota.

We studied four major classes of antibiotics used in agriculture (for veterinary purposes or as growth promoters) as well as in human medicine. We conducted 12 sampling campaigns over a 28-month period from 2011 – 2013, a time period that included extremely wet and dry seasons and therefore highly variable water levels. We collected samples from two agricultural drainage ditches, two municipal wastewater treatment plants, four locations in the river (upstream of both treatment plants, between the two plants, at the outfall of the second plant, and downstream of both plants), and from a nearby reference creek site. For collected samples we quantified six antibiotic resistance genes, susceptibility of cultivable bacteria to four antibiotics, and concentrations of six antibiotics.

The highest levels of antibiotics and antibiotic resistance were consistently associated with the municipal wastewater treatment plant samples. In addition, tetracycline-resistant bacteria isolated from wastewater treatment plants were found to be much more likely (103 out of 124 isolates) than tetracycline-resistant bacteria isolated from the river (0 out of 148 isolates) to have an integron, a mobile genetic element that can be associated with multiple-antibiotic resistance. These findings suggest human sources are much more significant than agricultural sources for this portion of the Minnesota River.

Project Results Use and Dissemination

The students who have been involved in this project have made multiple poster presentations in local venues on their work over the course of the project. In addition, the results have been disseminated via the following poster and oral presentations at professional conferences:

Wammer, K.H.; Beck, E.W.; Kapla, J.M.; Moffatt, M.E.; Harmes, D.C.; Sorensen, M.; Young, P; LaPara, T.M.; Stoll, D.R. “Antibiotics and antibiotic resistance in surface waters impacted by agricultural and municipal inputs.” Poster presentation at the Gorden Research Conference, Environmental Sciences: Water”, June 20-26 2014, Plymouth, NH.

Kapla, J.M.; Moffatt, M.E.; Beck, E.W.; LaPara, T.M.; Wammer, K.H. “Quantifying cultivable antibiotic-resistant bacteria in surface waters.” Poster presentation at the American Chemical Society Spring 2014 National Meeting, March 16-20 2014, Dallas, TX.

Moffatt, M.E.; Beck, E.W.; Kapla, J.M.; Burch, T.R.; LaPara, T.M.; Wammer, K.H. “Quantifying bacterial resistance to antibiotics in Minnesota surface waters.” Poster presentation at the American Chemical Society Spring 2014 National Meeting, March 16-20 2014, Dallas, TX.

Wammer, K.H.; Mofatt, M.E.; Beck, E.W.; Burch, T.R.; LaPara, T.M.; Stoll, D.R. “Antibiotics and antibiotic resistance in a river impacted by agricultural and municipal inputs.” Presentation at the American Chemical Society Fall 2013 National Meeting, September 8-12 2013, Indianapolis, IN.

Stoll, D.R.; Harmes, D.C.; Witt, K. “Quantitation of trace level antibiotics in Minnesota River water and wastewater effluent using an automated, integrated online-SPE LC-MS/MS System”. Poster presentation at HPLC 2013, June 16-20 2013, Amsterdam, The Netherlands.

Moffatt, M.E.; Beck, E.W.; Burch, T.R.; LaPara, T.M.; Wammer, K.H. “Bacterial resistance to four classes of antibiotics in the Minnesota River.” Poster presentation at the American Chemical Society Spring 2013 National Meeting, April 7-11 2013, New Orleans, LA.

It is also anticipated that manuscripts currently in preparation will result in two peer-reviewed publications in scientific journals.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2011 Work Plan Final Report

Date of Status Update: 7/1/14
Date of Next Status Update: Final Report
Date of Work Plan Approval: 6/23/2011
Project Completion Date: 6/30/2014 **Is this an amendment request?** _No____

Project Title: Assessment of Minnesota River Antibiotic Concentrations

Project Manager: Kristine Wammer
Affiliation: University of St Thomas
Address: 2115 Summit Ave, OSS 402
City: St Paul **State:** MN **Zipcode:** 55105

Telephone Number: (651) 962-5574
Email Address: khwammer@stthomas.edu
Web Address: http://www.stthomas.edu/chemistry/faculty/wammer.htm

Location:

Counties Impacted: Blue Earth, Le Sueur, Nicollet

Ecological Section Impacted: Minnesota and Northeast Iowa Morainal (222M), North Central Glaciated Plains (251B)

Total ENRTF Project Budget:		
	ENRTF Appropriation $:	190,000
	Amount Spent $:	185,284
	Balance $:	4,716

Legal Citation: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 05e

Appropriation Language:
$95,000 the first year and $95,000 the second year are from the trust fund to the commissioner of natural resources for an agreement with Saint Thomas University in cooperation with Gustavus Adolphus College and the University of Minnesota to measure antibiotic concentrations and antibiotic resistance levels at sites on the Minnesota River.

I. PROJECT TITLE: Assessment of Minnesota River Antibiotic Concentrations

II. FINAL PROJECT SUMMARY:

While the presence of antibiotics in surface waters has received attention due to concerns about health or ecological impacts, major gaps still remain in our understanding of the scope and significance of this potential problem. The goal of this study was to address the question of whether human or agricultural sources of antibiotics and antibiotic resistant bacteria may be the most significant in surface waters impacted by both. We focused on drainage ditches that receive farm runoff and municipal wastewater treatment plant effluents as possible sources for a portion of the Minnesota River in Southern Minnesota.

We studied four major classes of antibiotics used in agriculture (for veterinary purposes or as growth promoters) as well as in human medicine. We conducted 12 sampling campaigns over a 28-month period from 2011 – 2013, a time period that included extremely wet and dry seasons and therefore highly variable water levels. We collected samples from two agricultural drainage ditches, two municipal wastewater treatment plants, four locations in the river (upstream of both treatment plants, between the two plants, at the outfall of the second plant, and downstream of both plants), and from a nearby reference creek site. For collected samples we quantified six antibiotic resistance genes, susceptibility of cultivable bacteria to four antibiotics, and concentrations of six antibiotics.

The highest levels of antibiotics and antibiotic resistance were consistently associated with the municipal wastewater treatment plant samples. In addition, tetracycline-resistant bacteria isolated from wastewater treatment plants were found to be much more likely (103 out of 124 isolates) than tetracycline-resistant bacteria isolated from the river (0 out of 148 isolates) to have an integron, a mobile genetic element that can be associated with multiple-antibiotic resistance. These findings suggest human sources are much more significant than agricultural sources for this portion of the Minnesota River.

III. PROJECT STATUS UPDATES:

Project Status as of January 2012:

We conducted three initial sampling trips: on July 6, August 8, and October 11, 2011. As part of Activity 1, bacteria were cultivated on two different types of growth media amended with the four target antibiotics, although not all antibiotics and sites were studied on each trip. One major challenge we encountered was some contamination of the solid media amended with three of the antibiotics (streptomycin, tetracycline, and tylosin). Our preliminary results to date show no major differences in the fraction of bacteria that are able to grow in the presence of a range of concentrations of streptomycin, tetracycline, and tylosin among the various sample sites. Sulfamethoxazole data has been a bit more variable with slightly elevated growth at some sites. Our effort to date on Activity 2 has been primarily focused on development of methods for detection and quantification of two of the four initial target compounds, namely tylosin and sulfamethoxazole. We have used solid-phase extraction followed by high performance liquid chromatography with tandem mass spectrometry for detection. To date we have detected sulfamethoxazole in several WWTP effluent samples at concentrations above 10 ppt. As part of Activity 3, undergraduate students were trained in the techniques of extracting and purifying genomic DNA from these samples and began extracting DNA from the collected samples. We anticipate the undergraduate students will soon be trained in the technique of quantitative real-time PCR.

Amendment Request (08/30/12):

We have been more successful than originally hoped at competing for internal funds to support University of St. Thomas students to work on this project. Therefore, we are running low on supply money to support their work, but have extra funds budgeted for personnel. This amendment request is

to move $8,000 from the "Personnel" budget to the "Equipment/Tools/Supplies" budget; both apply to Activity 1. A few examples of items (and their costs) used to complete the work that may be purchased include: HPLC columns ($400-$900 per column) and solvents (e.g. acetonitrile, $20 per L) used to facilitate analysis of antibiotic concentrations prior to addition to the growth media, petri dishes ($90 per case) for culturing bacteria on solid media plates, and filters ($3 per filter) needed to avoid contamination of antibiotic solutions. This change does not impact the activity description below. The Project Summary Budget and Attachment A have been revised to reflect the change; amount spent figures are as of June 30 2012, the last quarterly reconciliation. (Amendment approved by LCCMR staff on 9/10/2012.)

Project Status as of October 2012:

In the first summer/fall of this project, we conducted three sampling trips. Between October 2011 and April 2012, when we were unable to obtain water samples, we refined our methods for Activities 1 and 2 and made significant progress on extracting DNA and quantifying antibiotic resistance genes for Activity 3. Since April 2012, we have successfully completed six additional sampling trips. These have occurred during highly variable seasonal conditions, and water levels have also varied considerably (literally, flood to drought conditions; the drainage ditches actually went dry this fall, which no locals could remember happening in recent memory). While there is variability from trip to trip, we have enough data now to see some clear trends. Antibiotic concentrations are not significant in the drainage ditches impacted primarily by agricultural runoff. They are also usually low in the river itself. In samples associated with wastewater treatment plants (WWTPs), however, measurable concentrations of antibiotics are commonly found. Antibiotic resistance genes are similar; for each of the genes we are studying, we consistently see higher amounts in samples associated with WWTPs. It is usually harder to see significant differences among sites when cultivable antibiotic-resistant organisms are measured, but when elevated growth in the presence of antibiotics is observed, it is also associated with WWTP samples. We will conduct one more sampling trip this fall (with very low water levels) and we will then assess what additional samples will be required next spring to successfully complete the project.

Project Status as of March 2013:

We conducted one additional sampling trip this past fall. Over the winter months (when sampling at most sites is not possible), work related to Activities 1 and 2 has been primarily related to method development and data analysis. We intend to conduct a couple of more sampling trips in the spring or early summer of 2013 to make use of our improved methodologies. Significant progress was made in processing the DNA samples necessary to complete Activity 3; in fact, the original scope of work on that activity is essentially completed. Because there will be additional sampling trips, however, some work on DNA samples from these trips will be performed to complement the data related to the other two activities. The overall project will likely be completed by this summer.

Project Status as of October 2013:

We conducted two additional sampling trips this past summer. Analysis of culture-based results and antibiotic concentrations from these trips was successful; analysis of gene-based results is still being completed but also has been successful to date. Overall, the work on this project is now nearing completion. All tasks related to Activity 2 have been completed. Almost all of the remaining work involves measuring multiple antibiotic resistance for bacteria isolated on previous sampling trips.

Project Status as of March 2014:

We are working on the effort to measure multiple antibiotic resistance for tetracycline-resistant isolates obtained on previous sampling trips. This work is all that remains to be done before we complete this project no later than June 2014. At this point, we are about halfway done analyzing these isolates.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collect samples and quantify cultivable antibiotic-resistant organisms at targeted Minnesota 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 the 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 identify these organisms and determine their resistance to multiple antibiotics). Samples will be obtained from seven locations selected to allow comparison of primarily agricultural, primarily residential/industrial, and mixed inputs to the Minnesota River. In an initial sampling effort, at least two sets of samples will be obtained from each of the sites. Bacteria from these initial sample sets will be cultivated on two different growth media amended with a range of concentrations of each of four antibiotics: tetracycline, sulfamethoxazole (a sulfa drug), tylosin (a macrolide), and streptomycin (an aminoglycoside). For the main sampling effort at least five sets of samples will be obtained from each of the sites, varying seasonally and with rainfall events. Cultivable antibiotic-resistant bacteria will be enumerated from each sample. Resistant bacteria will also be isolated and tested for resistance to other classes of antibiotics.

Summary Budget Information for Activity 1:

ENRTF Budget: **$** 52,281
Amount Spent: **$** 51,147
Balance: **$** 1,134

Activity Completion Date:

Outcome	Completion Date	Budget
1. Cultivate bacteria from the initial sample sets on two different growth media (PYT80 for slow-growing and LB for fast-growing bacteria) amended with a range of concentrations of the 4 antibiotics: tetracycline, sulfamethoxazole (a sulfa drug), tylosin (a macrolide), and streptomycin (an aminoglycoside).	September 2011	$ 6,767
2. Enumerate antibiotic-resistant bacteria from all 7 sites for each main sampling event.	September 2012	$ 37,757
3. Isolate resistant bacteria, and test their resistance to other classes of antibiotics	December 2012	$ 7,757

Activity Status as of January 2012:

We conducted three initial sampling trips: on July 6, August 8, and October 11, 2011. For the first trip, we collected samples and cultured bacteria from six sites (Sites 1, 2, 5, 6, 8, and 9; see revised map). As planned, bacteria were cultivated on two different types of growth media amended with the four target antibiotics. Prior to the first trip we determined that it was not possible to detect decreased susceptibility to sulfamethoxazole on the LB media, so Isosensitest broth (ISB) media was substituted to measure fast-growing bacteria in the presence of sulfamethoxazole and will be for the remainder of the project. For the second trip, we collected samples and cultured bacteria from all 9 sites in the presence of all four antibiotics; for the third trip we collected samples from Sites 2,3,5,6,7, and 9 and grew bacteria on three antibiotics (tetracycline was excluded).

One major challenge we encountered was contamination in some cases of the solid media amended with three of the antibiotics (streptomycin, tetracycline, and tylosin). In the months since the

last sample trip we have worked on resolving this problem. We are confident now that our new techniques (a combination of filter sterilization and autoclaving) will minimize this problem and that once the river thaws in the spring we will be able to collect samples that are not compromised by contamination.

Despite this challenge, we still obtained some useful data from our initial sampling trips. So far, we see no major differences in the fraction of bacteria that are able to grow in the presence of a range of concentrations of streptomycin, tetracycline, and tylosin among the various sample sites. Sulfamethoxazole data has been a bit more variable. Slightly elevated growth in the presence of 10 µM and higher sulfamethoxazole concentrations was observed at some sites. These were drainage ditch sites in the summer, and WWTP effluent in the fall. These data are very preliminary, but we have had a successful start to the project. Our main sampling campaign will begin ~April 2012 or whenever the weather permits.

Activity Status as of October 2012:

During the time period since our last status report, we conducted an additional six sampling trips: on April 20, May 31, June 11, July 9, July 30, and August 27 2012. The first trip was conducted as soon as possible after the spring thaw, and samples were collected from 9 sample sites (listed from southwest to northeast, in the direction of river flow): 2 drainage ditches several miles northwest of Lake Crystal (Sites 8 and 9), the Minnesota River at Land of Memories Park in Mankato (Site 6), effluent from the Mankato wastewater treatment plant (WWTP) (Site 7), the Minnesota River at Seven Mile Creek (Site 4), Seven Mile Creek itself (a reference site, Site 5), effluent from inside the plant and from at the outfall at the Minnesota River for the St. Peter WWTP (Sites 2 and 3), and the Minnesota River downstream of St. Peter (Site 1). The May 31 trip was previously unplanned but was conducted because flooding of the area caused river levels to rise to extremely high levels. Due to lack of lead-time, we were unable to prepare media and obtain culture-based data on that trip, but did collect samples for antibiotic concentration and resistance gene data. For each of the subsequent four trips, samples were collected from all 9 sites; in addition St. Peter WWTP influent (Site 10) was collected.

Our methodology has been refined since our initial sampling trips were conducted in 2011. Our previous problems with contamination have been resolved; all antibiotics other than tetracycline have been found to be stable during autoclaving so can be added to the media prior to sterilization. Tetracycline must be added aseptically after autoclaving, so we are utilizing a filter sterilization technique. In the spring of 2012, we removed one of our original target antibiotics (streptomycin) from the study. It was replaced with a new antibiotic (ciprofloxacin) which we studied beginning with the July 9 trip. For all six trips completed in 2012, the remaining original three target antibiotics were studied: tetracycline, sulfamethoxazole, and tylosin.

For each of the trips (excluding May 31), bacterial growth was measured in the presence of four concentrations of each antibiotic on two different types of solid media (high nutrient and low nutrient), in addition to on antibiotic-free control solid media. In general, differences among sites are much less compelling for the culture-based data when compared to results for the gene-based data described below. The general findings, however, match what is observed both for the antibiotic concentrations and antibiotic resistance genes. When elevated levels of bacterial growth in the presence of antibiotics are observed, it is almost always in samples obtained from wastewater treatment plants (effluent and at the outfall).

Activity Status as of March 2013:

One additional sampling trip was conducted in October 2012; because the drainage ditches were dry, those sites were excluded from this trip. Growth in the presence of four antibiotics on two media types was measured. Most work related to Activity 1 over the late fall and winter has been related to analysis of data from all previous sampling trips, and refinement of methods in preparation for additional sampling trips planned for this spring/early summer.

Activity Status as of October 2013:

Two additional sampling trips were conducted in Summer 2013. It is expected that these will be the final two sampling trips related to this project. For samples collected on these trips, growth in the presence of the four antibiotics (ciprofloxacin, sulfamethoxazole, tetracycline, and tylosin) on two media types was measured. Samples were collected from all nine sites on both trips. Implementation of improved methodologies since late summer 2012, in addition to having enough data to combine results from several trips, makes it much easier now to draw conclusions from this culture-based data. Although data variability is much higher than with the gene-based data associated with Activity 3, it is clear that when elevated growth is observed in the presence of an antibiotic, it is almost always associated with wastewater treatment plant samples. Our primary focus now related to this activity is analyzing bacteria isolated from previous samples for multiple antibiotic resistance. Approximately 400 tetracycline-resistant organisms have been isolated, and as many unique isolates as possible are now being tested for resistance not only to the four antibiotics specified previously, but also to ampicillin, polymixins, trimethoprim, rifampicin, and streptomycin.

Activity Status as of March 2014:

We have continued to analyze tetracycline-resistant organisms isolated previously for multiple antibiotic resistance. Our plan at this point is to analyze approximately 200 isolates, and we have currently completed work on approximately half that number. This is the last work that needs to be done before this project draws to a close in June 2014.

Final Report Summary:

A total of 12 sampling trips were completed between 2011 and 2013. Early trips were primarily devoted to method development; quantifiable data was obtained beginning in 2012. The fraction of all cultivable bacteria able to grow on solid media amended with each of four antibiotics (sulfamethoxazole, ciprofloxacin, tylosin, and tetracycline) was compared for a total of nine sample sites. Culture-based techniques provide highly variable data, and significant variations in water levels over the course of the sampling campaign created challenges for obtaining reliable data. Nevertheless, despite high data variability, consistent trends were observed. Bacteria associated with municipal wastewater treatment plant samples were typically more resistant to antibiotics than bacteria from agricultural drainage ditches or the Minnesota River itself. Tetracycline-resistant bacteria isolated from wastewater treatment plant effluents were also very much more likely to possess integrons than tetracycline-resistant bacteria isolated from the river. When these isolates were screened for multiple antibiotic resistance, however, there was not a clear and consistent difference between the effluent and river isolates. Therefore, while bacteria associated with wastewater treatment plant effluents are more likely than river bacteria to be resistant to antibiotics and much more likely to possess mobile genetic elements that are associated with lateral gene transfer, no evidence was observed that they are also significantly more likely to be resistant to multiple antibiotics.

Some funds for Activity 1 and Activity 3 were not expended. This is because we were more successful than originally anticipated at competing for internal university funds to support undergraduate students performing work related to this project. Therefore, while some funds remain, all the proposed work was still completed.

ACTIVITY 2: Measure antibiotic concentrations at same Minnesota River sites

Description: Samples from the initial sampling effort will be screened for the presence of the same four antibiotics listed in Activity 1. Based on these results, antibiotic detection methods will be optimized for the site matrices. New target antibiotics may also be added based on these results (especially if original target molecules are not detected.) Concentrations of the original and any other identified target molecules will then be measured in each sample collected as part of the main sampling effort. We will analyze water samples for the presence of selected antibiotics using methods based on high performance liquid chromatography (HPLC) that have recently been 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. For example, the Stoll group has successfully measured the concentration of phenytoin (a commonly used antiepileptic drug) in St. Peter, MN wastewater treatment plant effluent. We will use established solid-phase extraction (SPE) methods for sample pre-concentration prior to analysis to allow detection of antibiotics present at low levels in the river water and treatment plant effluent samples.

Summary Budget Information for Activity 2:

ENRTF Budget: **$** 56,938
Amount Spent: **$** 56,938
Balance: **$** 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Collect at least 2 sets of samples and screen them for the presence of the 4 target antibiotics.	September 2011	$ 10,441
2. Optimize our methods for the site matrices based on initial samples.	January 2012	$ 6,357
3. Collect at least 5 additional sample sets by the end of September 2012. Quantify concentrations of the target antibiotics at all 7 sites for each sampling event.	June 2013	$ 40,140

Activity Status as of January 2012:

Our effort to date has been primarily focused on development of methods for detection and quantification of two of the four initial target compounds, namely tylosin and sulfamethoxazole. We have used solid-phase extraction followed by high performance liquid chromatography with tandem mass spectrometry for detection. Initially we screened all of the samples collected by the Wammer group on the July 6 and August 8 sampling trips for the presence of tylosin and sulfamethoxazole, with an initial detection limit of about 50 parts-per-trillion (ppt). With the exception of sulfamethoxazole, which we have measured above 50 ppt in some WWTP effluent samples, we have not detected these two antibiotics in any other samples above this level. Thus, we elected to refine our methods to push the detection limits lower to 10 ppt for these compounds before moving on to develop methods for tetracycline and streptomycin. To date we have detected sulfamethoxazole in several WWTP effluent samples at concentrations above 10 ppt. In the next few months we will aim to develop similarly capable methods for tetracycline and streptomycin so that we are ready to screen the next set of samples collected by the Wammer group in the spring of 2012.

Upon the initial finding of elevated resistance of bacteria in one of the drainage ditch samples to sulfamethoxazole, which was not detected in the same sample at or above 10 ppt, we also developed methods to detect sulfachloropyridazine and sulfathiazole. In each case the method detection limit was about 10 ppt, however neither of these compounds was detected at or above this level in the drainage ditch samples.

Activity Status as of October 2012:

Our effort related to Activity 2 during this calendar year has been focused on continuing refinement of the analytical methodologies used for antibiotic detection and quantification, and processing approximately 100 water samples from several sites at different times. During this time period we consistently observed that water samples obtained from the Minnesota River channel contained very low levels of the antibiotics studied, ranging from ‘not detected’ (i.e., concentrations were below our method detection limits in the 10 ppt range) to concentrations below 50 ppt. However, we have consistently observed a few antibiotics in samples of urban wastewater effluent, some of which have been present at concentrations as high as 5 ppb. Most recently we have worked to transition from offline sample extraction to an analytical methodology that integrates the sample extraction process into the quantitative separation and detection step (LC-MS/MS). This improved

methodology will improve sample throughput, reduce sample carryover and losses, and improve the precision and accuracy of our quantitative data in support of Activity 2.

Activity Status as of March 2013:

Our effort related to Activity 2 since October 2012 has been focused on the refinement of an approach to online SPE that integrates most aspects of the analysis and minimizes sample handling. This is particularly beneficial for some classes of analyses (e.g., tetracyclines) that adsorb strongly to surfaces, leading to low recoveries and inaccurate quantitation. The improved recovery of the online SPE approach has revealed the presence of several antibiotics at sub-10-ppt levels in locations where we had not ever observed them in the past. Currently we are working to increase the robustness of this approach by developing novel inline filtration methods to further minimize sample handling. This development will support reliable operation of the analytical system for analysis of tens of samples at a time.

Activity Status as of October 2013:

Our effort related to Activity 2 since April 2013 focused on improving the robustness of the online-SPE-LC-MS/MS analysis approach we have developed for the quantitative determination of antibiotics in water samples. During this time we have analyzed approximately 75 water samples. Specifically, have developed a simple inline filtration approach that removes large, low density particulate matter that remains suspended in water samples following centrifugation. Also, we have improved the robustness of MS/MS detection by implementing both two dimensions of HPLC separation prior to detection, and a polarity switching approach that mitigates detector drift due to accumulation of ionic material in the mass spectrometer over time. We have developed a reliable quantitative approach that requires minimal sample handling and processing, and enables accurate quantitation by properly handling sample matrix effects that vary significantly across water sampling sites. This part of the project is now complete; a summary of findings will be reported as part of the final report summary.

Activity Status as of March 2014:

Activity 2 was complete as of the last status report; there is no new work to report.

Final Report Summary:

Methods to detect antibiotics in water samples were refined throughout the project period. One of the most significant developments was addition of an online SPE component coupled with LC-MS/MS analysis. A two-dimensional LC method was also used for the final two sample trips in 2013. The developed methods enabled reliable quantitation of antibiotic concentrations in complex samples including river water and effluent by mitigating sample matrix effects. Detection limits for some antibiotics were as low as the single digit parts per trillion range; quantification limits for all antibiotics were 50 ppt or lower. Target antibiotics included tetracycline, sulfamethoxazole, chlortetracycline, ciprofloxacin, tylosin, and erythromycin.

Similar to the patterns that were observed for antibiotic-resistant bacteria in Activity 1, elevated antibiotic concentrations were primarily associated with municipal wastewater samples. When seasonal conditions led to low water levels, trace concentrations of some antibiotics were occasionally detected in the river, however always at lower levels than those measured in wastewater influent and effluent.

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, sulfa, macrolide, and aminoglycoside classes.

Summary Budget Information for Activity 3:

ENRTF Budget: **$** 80,781
Amount Spent: **$** 77,199
Balance: **$** 3,582

Activity Completion Date:

Outcome	Completion Date	Budget
1. Quantify genes conferring resistance to tetracyclines, sulfa drugs, macrolides, and aminoglycosides in bacteria from the initial sample sets.	April 2012	$ 28,273
2. Quantify genes conferring resistance to the 4 classes of antibiotics at all 7 sampling sites for each sampling event.	June 2013	$ 52,508

Activity Status as of January 2012:

Three different sets of samples were properly collected and preserved during this period of effort. Undergraduate students were trained in the techniques of extracting and purifying genomic DNA from these samples; as such, the process of extracting and purifying DNA from the samples is on-going. We anticipate the undergraduate students will soon be trained in the technique of quantitative real-time PCR and will be generating significant quantities of useful data in the next reporting period.

Activity Status as of October 2012:

Genomic DNA from all of the samples collected prior to July 1, 2012 have been extracted, purified, and preserved. Quantitative real-time PCR has been used to enumerate number antibiotic resistance genes all of these samples (*intI1*, *tet*(A), *tet*(X), *tet*(W), *ermB*, *sul1*). Additional samples have been collected since July 2012; the processing of these samples (genomic DNA extraction and quantitative real time PCR) is on-going. We are also simultaneously developing a technique to quantify resistance to fluoroquinolones (*qnrA*).

Activity Status as of March 2013:

Genomic DNA from all samples have been extracted, purified, and preserved. Quantitative real-time PCR has been used to enumerate the number of antibiotic resistance genes in all of these samples (*intI1, tet*(A)*, tet*(X)*, tet*(W)*, ermB, sul1, qnrA)*. All work is complete as of this writing, although additional samples will likely be collected for the first two activities; additional quantitative PCR will be performed to complement these activities.

Activity Status as of October 2013:

Genomic DNA from all samples collected during summer 2013 has been extracted, purified, and preserved. Quantitative real-time PCR analysis of antibiotic resistance genes in all of these samples is being performed currently; this work is nearing completion. In addition, work is being done to support the multiple antibiotic resistance analysis being performed as part of Activity 1. Tetracycline-resistant isolates have been analyzed for the presence of integrons (mobile genetic elements that can be associated with multiple antibiotic resistance), and ribosomal intergenomic spacer analysis (RISA) is being done to help identify unique isolates for further testing. This part of the project is also nearing completion.

Activity Status as of March 2014:

This activity is not completely finished; some experiments in support of the multiple antibiotic resistance work of Activity 1 will be completed prior to the end of the project period. However, most of Activity 3 has been completed and no work or expenses related to Activity 3 are being reported since the last activity status update.

Final Report Summary:

Genomic DNA was extracted and purified from all collected samples. Quantitative PCR was used to quantify 16s rRNA (as a measure of total bacterial numbers), a Class I integron (*intI1*), and six antibiotic resistance genes including three tetracycline resistance genes (*tet(A), tet(X), tet(W)*), one macrolide resistance gene (*ermB*), one sulfonamide resistance gene (*sul1*), and one quinolone resistance gene (*qnrA*). 16s rRNA data showed that bacterial levels were fairly consistent across sample sites; the amount of bacteria present in effluent from the wastewater treatment plants was typically no higher than the receiving waters. However, similar to the results from Activity 1, the quantity of antibiotic resistance genes was consistently higher in samples associated with wastewater treatment plants than in river or drainage ditch samples. In some cases, quantities of antibiotic resistance genes were attenuated during the treatment process; effluents had lower concentrations than influents. In other cases, significant removal did not occur during the treatment process.

V. DISSEMINATION:

Description: The results of this study will be disseminated through oral and poster presentations by the students involved in the project, briefings to LCCMR as requested, and peer-reviewed publication. We will also present progress on the 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 2012: Because we are in an early stage of the project, it is too early to submit any results for publication. University of St. Thomas students did present two posters describing preliminary findings of the project at the University of Minnesota Summer Undergraduate Research Expo (August 11, 2011). The posters were "Determining Current Sulfamethoxazole and Streptomycin Resistance Levels in the Minnesota River", presented by Wendy Consoer and Sam Jensen, and "Significance of Antibacterial Resistance to Tetracycline and Tylosin in the Minnesota River", presented by Marianna Moffatt and James Byrnes.

Status as of October 2012: One University of St. Thomas undergraduate student (Marianna Moffatt) has submitted an abstract to present her work on this project at the Spring 2013 American Chemical Society (ACS) national meeting (April 7-11 2013, New Orleans, LA). The title of her presentation is "Bacterial resistance to four classes of antibiotics in the Minnesota River". UST co-authors on this presentation include Kris Wammer and another undergraduate student (Elizabeth Beck); UMN co-authors include Tim LaPara and a graduate student (Tucker Burch).

Status as of March 2013: Marianna Moffatt's abstract mentioned in the previous report was accepted; she will present a poster based on her work on April 8, 2013 at the ACS National Meeting in New Orleans. Kris Wammer has submitted an abstract to present a talk titled "Antibiotics and antibiotic resistance in a river impacted by agricultural and municipal inputs" at the Fall 2013 ACS national meeting (September 8-12 2013, Indianapolis, IN) as a part of the session "Distribution and Fate of Emerging Contaminants in Hydrologic Systems of the Built Environment." Co-authors include Tim LaPara and Dwight Stoll, UST students Elizabeth Beck and Marianna Moffatt, and UMN student Tucker Burch.

Status as of October 2013: Marianna Moffatt presented her poster at the Spring 2013 ACS National meeting in New Orleans, and Kris Wammer presented her talk at the Fall 2013 ACS national meeting in Indianapolis (both mentioned previously as submissions). Dwight Stoll presented a poster titled "Quantitation of trace level antibiotics in Minnesota River water and wastewater effluent using an automated, integrated online-SPE LC-MS/MS system" at HPLC 2013 in Amsterdam, the Netherlands, in June 2013.

Status as of March 2013: Marianna Moffatt and Jacqueline 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.

Final Report Summary:

The students who have been involved in this project have made multiple poster presentations in local venues on their work over the course of the project. In addition, the results have been disseminated via the following poster and oral presentations at professional conferences:

Wammer, K.H.; Beck, E.W.; Kapla, J.M.; Moffatt, M.E.; Harmes, D.C.; Sorensen, M.; Young, P; LaPara, T.M.; Stoll, D.R. "Antibiotics and antibiotic resistance in surface waters impacted by agricultural and municipal inputs." Poster presentation at the Gorden Research Conference, Environmental Sciences: Water", June 20-26 2014, Plymouth, NH.

Kapla, J.M.; Moffatt, M.E.; Beck, E.W.; LaPara, T.M.; Wammer, K.H. "Quantifying cultivable antibiotic-resistant bacteria in surface waters." Poster presentation at the American Chemical Society Spring 2014 National Meeting, March 16-20 2014, Dallas, TX.

Moffatt, M.E.; Beck, E.W.; Kapla, J.M.; Burch, T.R.; LaPara, T.M.; Wammer, K.H. "Quantifying bacterial resistance to antibiotics in Minnesota surface waters." Poster presentation at the American Chemical Society Spring 2014 National Meeting, March 16-20 2014, Dallas, TX.

Wammer, K.H.; Mofatt, M.E.; Beck, E.W.; Burch, T.R.; LaPara, T.M.; Stoll, D.R. "Antibiotics and antibiotic resistance in a river impacted by agricultural and municipal inputs." Presentation at the American Chemical Society Fall 2013 National Meeting, September 8-12 2013, Indianapolis, IN.

Stoll, D.R.; Harmes, D.C.; Witt, K. "Quantitation of trace level antibiotics in Minnesota River water and wastewater effluent using an automated, integrated online-SPE LC-MS/MS System". Poster presentation at HPLC 2013, June 16-20 2013, Amsterdam, The Netherlands.

Moffatt, M.E.; Beck, E.W.; Burch, T.R.; LaPara, T.M.; Wammer, K.H. "Bacterial resistance to four classes of antibiotics in the Minnesota River." Poster presentation at the American Chemical Society Spring 2013 National Meeting, April 7-11 2013, New Orleans, LA.

It is also anticipated that manuscripts currently in preparation will result in two peer-reviewed publications in scientific journals.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget:

Budget Category	$ Amount	Explanation
Personnel:	$53,002	$20,336 for principal investigator (Wammer), which includes 1.5 months of summer salary per year plus associated fringe benefits. $32,666 for undergraduate students: two working full-time each summer and three working 10 hours per week during the academic year.
Professional/Technical Contracts:	$ 120,650	$63,712 to University of Minnesota (LaPara) which includes 5 weeks of summer salary per year plus associated fringe benefits ($35,000), lab supplies

		($9,575), and services ($19,137). $56,938 to Gustavus Adolphus College (Stoll) includes 0.9 month of summer salary per year plus associated fringe benefits ($11,881), a research technician working 20 hours per week ($27,873), one student working full time each summer ($10,714), lab supplies ($3,170), instrument access ($3,000), and travel ($300).
Equipment/Tools/Supplies:	$14,598	General lab supplies for measuring antibiotic concentrations and culturing bacteria, e.g. antibiotics, nutrient media components, petri dishes, pipettes and tips, HPLC supplies
Travel Expenses in MN:	$ 1,750	Mileage reimbursement and meals for 9-10 total sampling trips
TOTAL ENRTF BUDGET:	**$ 190,000**	

Explanation of Use of Classified Staff: Summer 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: 2 FTE per year

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**	

*Note: Actual other funds exceeded the amount proposed.

VII. PROJECT STRATEGY:

A. Project Partners:

Kristine Wammer, University of St. Thomas, Department of Chemistry ($69,350; includes salary, students, general supplies, and travel)

Dwight Stoll, Gustavus Adolphus College, Department of Chemistry ($56,938; includes salary, research technician and students, general supplies, instrument access, and travel)

Timothy LaPara, University of Minnesota, Department of Civil Engineering ($63,712; includes salary, supplies and services)

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. By focusing on the selected portion of the Minnesota River, we will be able to investigate the relative importance of agricultural vs. municipal inputs. This work will inform future regulations related to

wastewater, drinking water, and agriculture, and the advisability of the ever-increasing practice of using treated wastewater for non-potable applications (this is known as “water reuse”).

C. Spending History: none

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. MAP(S): N/A

X. RESEARCH ADDENDUM: See Research Addendum

XI. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted not later than January 2012, October 2012, and March 2013. A final report and associated products will be submitted between June 30 and August 1, 2013 as requested by the LCCMR.

Final Attachment A: Budget Detail for M.L. 2011 (FY 2012-13) Environment and Natural Resources Trust Fund Projects

Project Title: Assessment of Minnesota River Antibiotic Concentrations

Legal Citation: $95,000 the first year and $95,000 the second year are from the trust fund to the commissioner of natural resources for an agreement with Saint Thomas University in cooperation with Gustavus Adolphus College and the University

Project Manager: Kristine Wammer

M.L. 2011 (FY 2012-13) ENRTF Appropriation: $ 190,000

Project Length and Completion Date: 2 years; June 30, 2013

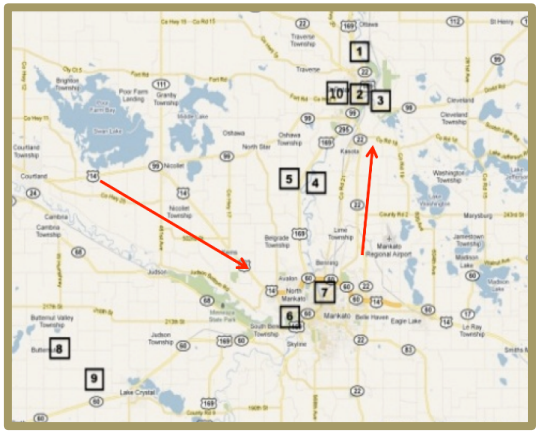
Date of Update: July 1, 2014

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 Minnesota River sites			Measure antibiotic concentrations at same Minnesota River sites			Quantify antibiotic resistance genes				
Personnel (Wages and Benefits)											
Kristine Wammer, Project Manager: $20,336 (93% salary, 7% benefits), 1.5 summer months effort	$20,336.00	$20,182.12	$153.88							$20,336	$154
Undergraduate Research Assistants: $40,666 (96% salary, 4% benefits), 3 students full-time for 12 weeks each summer. 2 students working 10 hours for 32 weeks during the academic year.	$15,597.00	$14,816.88	$780.12				$17,069.00	$13,486.49	$3,583	$40,666	$4,363
Professional/Technical Contracts											$0
University of Minnesota: Timothy LaPara, Principal Investigator. 5 weeks of summer salary per year plus associated fringe benefits. Duties: Responsible for gene-based tests of antibiotic resistance, including supervision of St. Thomas undergraduate students (Activity 2) ($35,000). General lab supplies, e.g. PCR primers, reagents ($9,575). Services associated with gene sequencing ($19,137)							$63,712.00	$63,712.00	$0	$63,712	$0
Gustavus Adolphus College: Dwight Stoll, Principal Investigator. 0.9 month of summer salary per year plus associated fringe benefits. Duties: Responsible for analysis of concentrations of antibiotics and supervision of Gustavus undergraduate students and research technician (Activity 1) ($11,881). Research technician 20 hours per week at $12 per hour plus associated fringe benefits ($27,873). 1 student during each summer, 40 hours per week for 12 weeks each year, $10 per hour, plus associated fringe benefits ($10,714). General lab supplies, e.g. solvents, vials, analytical standards ($3,170). LC/MS instrument access ($3,000). Travel for				$56,938.00	$56,938.00	$0.00				$56,938	$0
Equipment/Tools/Supplies											$0
General supplies, e.g. antibiotics, nutrient media, plates, etc.	$14,598.00	$14,508.14	$89.86							$6,598	$90
Travel expenses in Minnesota Mileage and meals for approximately 10 sampling trips.	$1,750.00	$1,640.16	$109.84							$1,750	$110
COLUMN TOTAL	**$52,281.00**	**$51,147.30**	**$1,133.70**	**$56,938.00**	**$56,938.00**	**$0.00**	**$80,781.00**	**$77,198.49**	**$3,582.51**	**$190,000.00**	$4,716

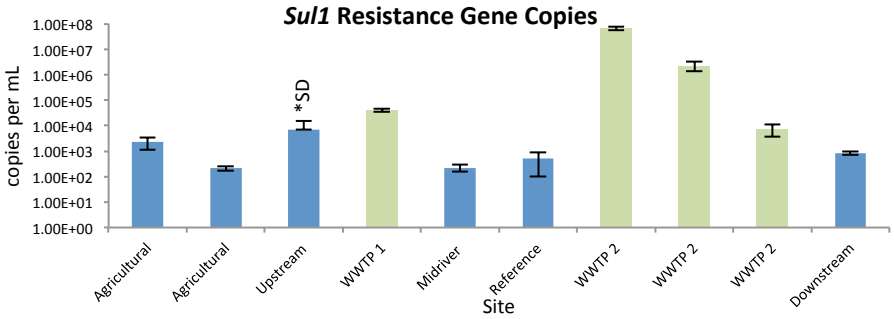
Assessment of Minnesota River Antibiotic Concentrations

The study was designed to identify any major sources of antibiotics and antibiotic resistance by collecting water samples at sites along the Minnesota River and performing the types of measurements shown.

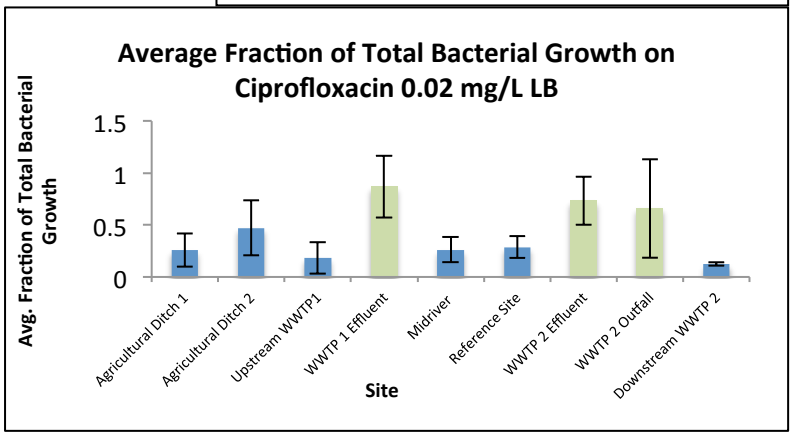
Site 1: Downstream of WWTP 2
Site 2: WWTP 2 Effluent
Site 3: WWTP 2 Outfall
Site 4: Between WWTP 1 and 2
Site 5: Reference Site
Site 6: Upstream of WWTP 1
Site 7: WWTP 1
Site 8: Agricultural Ditch 1
Site 9: Agricultural Ditch 2
Site 10: WWTP 2 Influent



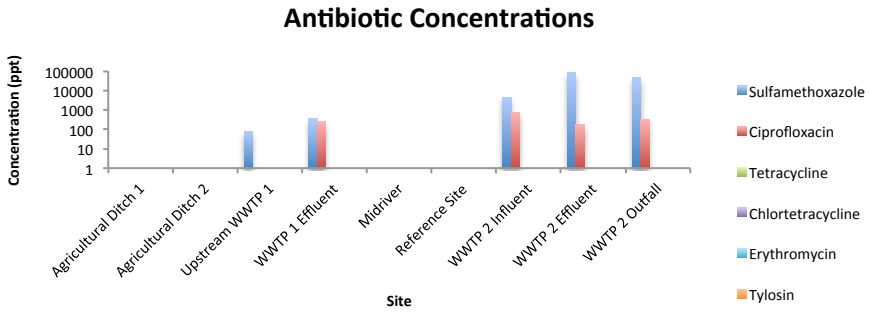
Antibiotic-Resistance Genes



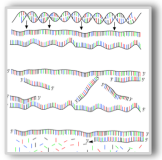
Antibiotic-Resistant Bacteria



Antibiotic Concentrations



Integrons



PCR-> Integron Present?

Kirby-Bauer Susceptibility Test -> Multiple Antibiotic Resistance?