Environment and Natural Resources Trust Fund

Research Addendum for Peer Review

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Project Title: Minnesota River: Occurrence and Potential Significance of Antibiotics

Project number: 030-B

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1. Abstract

Pharmaceuticals, including antibiotics, have recently been identified as emerging environmental contaminants. While numerous studies have reported the occurrence of antibiotics at low concentrations in natural waters, the significance of this problem as a potential ecological or human health concern is largely not understood. The goal of this project is to examine the potential threat of enhanced antibiotic resistance due to the presence of antibiotics in the Minnesota River. In particular, we will perform an initial assessment of the relative contributions of farm runoff and wastewater effluents to antibiotic concentrations and levels of antibiotic resistance among Minnesota River bacteria. To assess levels of antibiotic resistance, two complimentary approaches will be used; we will measure both cultivable antibiotic resistant bacteria and antibiotic resistance genes. This project will provide valuable information necessary to inform risk assessments, treatment options, and regulatory decisions concerning antibiotics in Minnesota waters.

2. Background

Antibiotics and Other Pharmaceuticals in the Environment

Pharmaceuticals and personal care products (PPCPs) have gained significant attention in recent years as emerging contaminants in the environment. The use of pharmaceuticals is extensive and increasing. According to a recent review (Khetan *et al.* 2007), 44% of Americans took at least one prescription drug, and 17% took three or more in 1999-2000. Annual pharmaceutical sales for human medicine in the United States are currently $248 billion with an annual growth rate of over 8%, almost seven times the population growth rate. Veterinary use of pharmaceuticals, in particular antibiotics, is also significant. In the United States, there are currently approximately 104-110 million cattle, 7.5-8.6 billion chickens, 60-92 million pigs, and 275-292 million turkeys (Sarmah *et al.* 2006), many in large confined animal feeding operations (CAFOs). A growing use of veterinary antibiotics for these large numbers of food animals is for growth promotion (low levels of the drugs are included in the animal feed) rather than disease treatment, with an 80-fold increase in use for growth promotion in the United States over the past forty years (Sarmah *et al.* 2006).

Significant fractions of many of these pharmaceutical compounds are resistant to metabolic degradation in the body and can be excreted in an active form (Halling-Sorenson *et al.* 1998, Daughton *et al.* 1999). Therefore, given the usage rates, it is not surprising that environmental monitoring efforts over the past several years have detected PPCPs in many locations, including various surface waters. Both human and agricultural sources have been implicated in these studies. For example, Calamari *et al*. surveyed the Po and Lambro rivers in Italy, in a highly populated area with several animal farms, and found several drugs at concentrations ranging from 0.1 to 250 ng/L (Calamari *et al.* 2003). Tixier *et al.* measured concentrations in the µg/L range for six drugs in three wastewater treatment plant (WWTP) effluents, and tracked variation over time in the effluents, two rivers, and a lake in Switzerland (Tixier *et al.* 2003). One study in particular that has stimulated considerable interest was a national survey of selected United States streams by the United States Geological Survey (Kolpin *et al*. 2002). This study has been cited over 1700 times since its publication eight years ago, making it the most cited paper in the history of the journal *Environmental Science and Technology*. The authors searched for 95 compounds including 22 antibiotics, of which 14 were detected at sub-therapeutic levels. Typical median concentrations were 0.05-0.5 µg/L with the highest concentration reported being 1.9 µg/L (sulfamethoxazole). The classes of detected antibiotics included the tetracyclines, sulfonamides, fluoroquinolones, and macrolides. With overall use of PPCP compounds, including antibiotics, on the rise, occurrence of these contaminants in surface waters and other environmental compartments is anticipated to continue into the foreseeable future.

Human and Agricultural Sources of Antibiotics: Response and Need for More Information

There have been several studies, spurred by reports of PPCP occurrence, that have succeeded in closely linking concentrations of human pharmaceuticals in receiving waters with loads from WWTP effluents (e.g. Ternes 1998). This has gotten the attention of lawmakers, and legislation has been proposed both at the state and national levels to regulate aspects of disposal of pharmaceuticals. For example, the MN House and Senate recently passed a bill (H.F. 1217, S.F. 1568) addressing who may legally handle pharmaceuticals for disposal. Researchers have also examined the potential significance of WWTPs for removal of human pharmaceuticals and found that the fraction of PPCPs that pass through various WWTP processes is highly variable by drug class (Gobel *et al*. 2007, Lindberg *et al.* 2005, Vieno *et al.* 2005, Miao *et al.* 2004, McArdell *et al.* 2003). In recent years, the potential has been examined for advanced treatment processes more typically used in drinking water treatment (such as ozonation, granular activated carbon (GAC), and advanced oxidative processes (AOPs)) to reduce WWTP pharmaceutical loads to the environment. Work done to determine fate of PPCPs in drinking water treatment plants has found many of these techniques effective in eliminating some of these classes of contaminants (Vieno *et al.* 2007, Stackelberg *et al.* 2004, Huber *et al.* 2003, Ternes *et al.* 2002). Discussions about whether regulatory actions and/or aggressive treatment strategies may be desirable or necessary, however, are hampered by lack of information concerning impacts of antibiotics and other PPCPs in the environment. In other words, while it is known these compounds are and will likely continue to be present at low levels in some natural waters, and that WWTP effluents are a potentially important source, it has not yet been established how significant of a problem this actually is.

Lawmakers and regulators have also taken notice of the occurrence in natural waters of antibiotics that likely originate from agricultural sources. The use of antibiotics for growth promotion has received the most attention as a cause for concern; the Preservation of Antibiotics for Medical Treatment Act of 2007 (S. 549, H.R. 962) sought to ban nontherapeutic use of seven classes of antibiotics that are important in human medicine: penicillins, tetracyclines, macrolides, lincosamides, streptogramins, aminoglycosides, and sulfonamides. Similar acts were previously considered in 2003 and 2005 but never made it out of committee. An updated version of this act (S. 619, H.R. 1549) currently under consideration instead merely seeks to require the Food and Drug Administration (FDA) to re-review the approvals of these seven classes of drugs for animal feed within two years of enactment, and rescind approval for those deemed unsafe from the standpoint of concerns about the development of antibiotic resistance. The FDA has also recently indicated they may create regulations independent of a congressional mandate. More fate and effects data is needed to inform such regulatory decisions; many current discussions are forced to rely on hypotheses and "what-if" scenarios. As stated in the conclusion of a recent review of antibiotics in the aquatic environment (Kümmerer 2009b): "There is still a lack of fundamental data on the fate and effects of antibiotics and antibiotic resistance genes in the environment. The availability of such data is a prerequisite if proper risk assessment and risk management programs for both humans and the environment are to be undertaken."

Detection of Antibiotics: Analytical Challenges

Determination of antibiotic concentrations in environmental water and WWTP effluent samples is challenging for a variety of reasons. Chief among these are the relatively low concentrations expected, especially since they are in the presence of thousands of other compounds, many of which are present at higher concentrations. This leads to serious ion suppression effects when electrospray ionization mass spectrometry is used for detection after separation of the target compounds from the sample matrix, which causes significant problems in quantification. Normally this problem is overcome through the use of stable isotope labeled internal standard compounds, however for many common antibiotics of interest, such standards are either not commercially available, or are prohibitively expensive. Other difficulties are encountered during water sampling and sample pretreatment, such as adsorption of target compounds to sampling containers and binding of target compounds to sample matrix constituents. Fortunately, the latter problems are well studied and solutions exist for most common antibiotics of interest.

Environmental Impacts of Antibiotics: Antibiotic Resistance

The ecological impacts of antibiotics in the environment have yet to be characterized; this issue, therefore, is controversial among scientists, leading to a muddled set of policy initiatives. While evidence to date suggests that most antibiotic levels are likely too low to have adverse ecotoxicity effects (Jones *et al.* 2004), there is some limited information in the literature suggesting harmful effects at environmentally-relevant concentrations (Thiele-Brune *et al.* 2005). Recent reviews summarize the current state of knowledge for many antibiotics and other human pharmaceuticals (Fent *et al.* 2006) and antibiotics in the aquatic environment (Kümmerer 2009a, Kümmerer 2009b), and point out the need for much more data in this area.

Further complicating the matter is that virtually all sources of free antibiotics in the environment (e.g., municipal wastewater or runoff from agricultural activities) are simultaneously sources of antibiotic resistant bacteria (i.e., fecal material from humans and/or animals).

The main concern associated with long-term exposure to antibacterial compounds is the proliferation of antibiotic resistance among benign environmental bacteria (Rooklidge 2004, Kümmerer 2004). Resistant environmental bacteria are of concern because they may serve as a reservoir for antibiotic resistance genes that can potentially be transferred to pathogenic strains. Overall, the potential for the low, subtherapeutic antibiotic concentrations that are found in natural waters to provide selective pressure for resistant organisms is not well-understood. While several studies have suggested a link between the presence of antibiotics and levels of antibiotic resistance genes in natural waters (e.g. Storteboom 2010a, Koike 2007), a recent study suggested that impacts of antibiotics on natural bacterial communities may be less significant than transportation of bacteria and antibiotic resistance genes from the source itself (Storteboom 2010b). In addition, several other studies have detected numerous antibiotic resistant bacteria in non-impacted surface waters (Jones *et al.* 1986, Alvero 1987, Pei *et al.* 2006, Pruden *et al*. 2006). A recent review summarizes the current state of knowledge and identifies the need for more information (Allen *et al.* 2010). Therefore, studies are needed to determine the extent to which the presence of low concentrations of antibiotics may be impacting natural microbial communities.

Research Needs and Project Goals

While the environmental occurrence of antibiotics and other PPCPs has spurred interest both in the research community and the public realm, major gaps still remain in our understanding of their significance and potential health and ecological impacts. Therefore, the critical questions of which antibiotics are of the most concern and which sources (human vs. agricultural) are the most significant are still largely unanswered. The goal of this project is to identify classes of antibiotics that pose a potential threat. We will particularly focus on the development of antibiotic resistance due to the presence of antibiotics in farm runoff and in wastewater treatment plant effluents, which then subsequently impact surface waters. This project will study the Minnesota River, which has significant agricultural and municipal inputs, providing the opportunity to examine the relative importance of each input type. The project will assess current antibiotic concentrations, current antibiotic resistance levels, and the potential for future increases in resistance levels. The study will include four major classes of antibiotics that are used both for growth promotion in agriculture and in human medicine: tetracyclines, sulfa drugs, macrolides, and aminoglycosides. A strength of this project is combining cutting-edge analytical chemistry techniques with rigorous microbiology and molecular biology techniques to characterize each site.

3. Hypotheses

This project is designed to allow us to test whether agricultural and/or municipal inputs of antibiotics have significant impacts on levels of antibiotic resistance among Minnesota River bacteria. We propose to test the following hypotheses:

1. The quantity of free antibiotic chemicals in surface waters will correlate to the quantity of antibiotic resistant bacteria in surface waters.

2. Treated municipal wastewater will be the primary source of both free antibiotic chemicals and antibiotic resistant bacteria (as detected by cultivation and antibiotic resistance gene detection).

The first hypothesis is of substantial importance because it will demonstrate that scientists need to be concerned about antibiotic resistant bacteria in the environment (currently of little concern) in addition to the ecological impact of free antibiotic chemicals in the environment (i.e., the current focus of scientific concern). The second hypothesis is pertinent because it will identify municipal wastewater (in spite of a relatively high degree of treatment) as a more prominent source of both antibiotics and antibiotic resistant bacteria. Currently, agriculture is typically identified as the primary source of antibiotics and resistant bacteria; our research, therefore, could lead to a paradigm shift in dealing with antibiotic resistance that is more effective than current strategies.

4. Methodology

Measure antibiotic concentrations at targeted Minnesota River sites

Site selection and sampling protocol

Samples will be obtained from seven sites that have been selected for analysis as part of this project (Sites A-G). These sites were selected to allow comparison of primarily agricultural (Sites A and B), primarily residential/industrial (Sites F and G), and mixed (Sites C, D, and E) inputs to the Minnesota River. Two sites (Sites A and B) are drainage ditches receiving input primarily from tile drains in agricultural fields. Dominant crop cover in the area is corn and soybean; there are several small hog operations immediately upstream of Site A and a few much larger hog operations immediately upstream of Site B. Manure from these hog farms is spread on the fields that drain into the drainage ditches. These drainage ditches discharge into Lily Lake and eventually Minneopa Creek, which then discharges into the Minnesota River just upstream of Mankato, a city of approximately 30,000 people. Three sites (Sites C, D, and E) are in the Minnesota River. Site C is immediately downstream of where Minneopa Creek feeds into the river, and immediately upstream of Mankato and where the Blue Earth River, a substantial tributary to the Minnesota River, feeds in. Site D is a few miles downstream of Mankato and upstream of St. Peter, a city of approximately 10,000 people. Site E is several miles downstream of St. Peter. Sites F and G are wastewater treatment plant effluents in Mankato and St. Peter, respectively, both of which are discharged into the Minnesota River. In addition to these seven primary sites, samples will be taken from Seven Mile Creek, which is between Mankato and St. Peter, not far from Site D. This site is expected to be more pristine than the other sites and will serve as a reference site (MPCA 2010). If possible, samples that are primarily influenced by manure runoff (as opposed to drainage ditches which are expected to be influenced by several sources, e.g. septic, manure) will also be obtained from a farm near Site A.

Initial sets of samples will be collected from each of the seven sites beginning in Summer 2011. More extensive sampling, varying with rainfall events and seasons, will commence in

Spring 2012. If unpredictable weather conditions preclude sampling of the selected drainage ditch sites, we will substitute samples from alternate ditches in the area and/or additional samples will be taken from other locations in Minneopa Creek. For each discrete sampling event, three sets of samples will be collected from each site, one each for analysis of antibiotic concentrations, quantification of culturable antibiotic-resistant bacteria, and analysis of antibiotic resistance genes. With the exception of samples collected for aminoglycoside analysis, samples for antibiotic concentration analysis will be collected in glass bottles that have been prewashed with EDTA solution to avoid binding of metals to the target antibiotics. At the time of sampling EDTA will also be added to the samples (to 0.05% (w/v)), and the solutions will be acidified to pH 3.0 by adding phosphoric acid (Batt, 2005). For aminoglycoside analysis, samples will be collected in polypropylene bottles, followed by addition of EDTA and phosphoric acid as described above. Samples for quantification of culturable antibiotic-resistant bacteria will be collected in clean polyethylene bottles that have been sterilized by autoclaving. At the sample site the bottles will be rinsed at least three times with the sample water prior to collection. Water samples will be collected and kept in a cooler on ice to maintain the bacterial community until the bacteria can be cultured. Samples for analysis of antibiotic resistance genes will be passed through a 47-mm nitrocellulose filter (pore size = 0.22 μm) to concentrate microbial biomass for subsequent genomic DNA extraction and purification; the quantity of water for this analysis will vary depending on the turbidity of the water (sample volumes will be recorded).

Quantification of four antibiotics

Prior to analysis by liquid chromatography coupled with tandem mass spectrometric detection (LC/MS/MS), samples will be pretreated using Solid Phase Extraction (SPE) to preconcentrate the target antibiotics and remove humic substances from the water sample. Hydrophilic/Lipophilic Balance (HLB) SPE material from Supelco will be used for all target compounds except aminoglycosides. After elution from the SPE cartridges, the samples will be evaporated to dryness, followed by reconstitution with an aqueous/organic solvent mixture prior to LC/MS. In the case of aminoglycoside analysis, a weak cation-exchange SPE material from Supelco (carboxypropyl) will be used for sample pretreatment.

For samples collected during the initial sampling effort we will use conventional LC/MS/MS methods with a triple quadrupole mass spectrometer to screen for the four initial antibiotic targets of the study, as well as any targets identified later in the project. At the time of collection we will spike these samples with $^{13}C_6$-sulfamethazine to serve as an internal standard for the entire quantitation process including sample pretreatment and analysis (Batt, 2005). For the analysis of aminoglycosides we will use Hydrophilic Interaction Liquid Chromatography (HILIC) columns and conditions as a means of dealing with the highly hydrophilic nature of these compounds. For the analysis of all other target antibiotics we will use conventional C18-type reversed-phase columns and conditions. In both cases we will take advantage of recent advances in HPLC column technology and use shell type particles (also known as fused-core) to obtain the highest chromatographic resolution and sensitivity possible with our instrumentation.

Prior to analysis of samples from the main sampling effort we will develop multidimensional methods for each of the target antibiotics identified in the initial sampling effort. Where

possible we will develop multi-target methods to minimize the total number of analyses required. As others have shown previously (Pascoe 2001, Hernandez 2005), and from our own recent results (Simpkins, 2010), the use of multidimensional separations (as opposed to conventional one-dimensional HPLC) prior to detection by electrospray MS/MS can significantly minimize sample matrix effects. This is especially valuable in situations when isotope labeled internal standards are not available for each of the target compounds (as is the case here), and when the expected target compound concentrations are low enough that ionization suppression could push the signal below the method detection limit. In this case we will again spike samples at the time of sampling with the $^{13}C_6$-sulfamethazine, but the internal standard will be used in this case primarily as a means of correcting for losses of target compounds in the sample preparation process. We will evaluate the extent of ionization suppression for each of the target analytes in the most complicated matrix (WWTP effluent) using the method of standards addition as a means of verifying that the multidimensional separation is effective at minimizing matrix effects. In addition to reducing ion suppression by increasing the resolving power of the chromatographic separation step, multidimensional chromatography methods will also increase the level of certainty in the identification of the antibiotic targets in unknown samples by providing additional time points (multiple chromatographic time axes instead of just one) that are characteristic of specific target compounds.

Measure antibiotic resistance levels at same Minnesota River sites

Quantification of culturable antibiotic-resistant bacteria

Bacteria collected from each sample will be cultivated on solid nutrient media within a few hours of collection using standard spread- and streak-plating techniques. Two types of solid media will be used to cultivate bacteria: LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per liter) and PYT80 agar (80 mg peptone, 80 mg yeast extract, 80 mg tryptone, and 15 g agar per liter). It is well known that the majority of bacteria do not grow on microbiological media. This approach, in which two types of growth media are used, has been previously shown to cultivate bacteria similar to those found in fecal material (LB agar) as well as those that appear to be indigenous to the natural environment (PYT80 agar) (Ghosh and LaPara, 2007).

Solid media will be amended with a range of concentrations (0 - 500 μM) of one of each of the four antibiotics listed previously: tetracycline, sulfamethoxazole, tylosin or streptomycin. Any additional antibiotics of interest, as identified during the course of the study, will also be included. Antibiotics will be added to the media aseptically after autoclaving. LB plates will be incubated in the dark at 37 °C whereas PYT80 plates will be incubated in the dark at 26 °C. Bacterial growth will be assessed by counting colony-forming units (CFUs) after bacterial growth is easily visible, which is typically on the order of 24 hours for LB plates and 72 hours for PYT80 plates. All bacterial enumerations will take place in triplicate. The amount of growth on plates with a given antibiotic concentration compared to the growth on plates with no antibiotic will be used to define what fraction of the bacterial community exhibits resistance to the antibiotic at that concentration.

Resistant bacterial colonies, i.e. those able to grow at relatively high concentrations of a given antibiotic, will be aseptically picked from the antibiotic-amended agar plates and repeatedly streaked to ensure that pure cultures are obtained. The colonies will then be grown in LB or PYT80 broth. Cells from these broth cultures will then be harvested for cryopreservation (storage in 15% glycerol at -70°C). Isolates will be analyzed for their resistance to multiple antibiotics (all four antibiotics included in this study, in addition to ciprofloxacin and ampicillin) using standard protocols used to determine minimum inhibitory concentrations (MICs) (Andrews 2001). In addition, we will look for the presence of 14 known genes that encode for tetracycline resistance in our isolates, as described in our previous work (Ghosh and LaPara, 2007).

Quantification of genes conferring antibiotic resistance

Real time PCR will be used to quantify the presence of a handful of different genes that code for antibiotic resistance (Table 1). We have selected these gene targets based on our prior experience and the availability of well-established quantitative PCR protocols in the published literature. We will assay three genes that encode resistance to tetracycline that represent each of the three known mechanisms of resistance (*tet*(A): efflux pump; *tet*(O): ribosomal protection protein; *tet*(X): enzymatic modification) (Chopra and Roberts, 2001). We will also target *erm*(B) and *erm*(T) genes, which encode resistance to macrolide antibiotics (tylosin, erythromycin, etc) because these genes were the most prominent in animal manure and animal treatment systems in a recent study (Chen *et al.*, 2007). We will likewise quantify the amounts of *sul1* (resistance to sulfa drugs). There is currently no available qPCR method for quantifying genes encoding resistance to streptomycin. As part of this study, we will either develop a novel method to quantify streptomycin resistance genes (we have done similar work for *tet*(X) (Ghosh et al. 2009)) or focus strictly on resistance to the other three classes of antibiotics. All samples will be normalized to the quantity of 16S rRNA genes, also measured by real time PCR.

Table 1. Gene targets, primer sequences, and annealing temperatures for real-time PCR assays to be used in the proposed study.

Gene	Primer sequence (5′→3′)	Expected Amplicon Size (bp)	Primer Annealing Temp (°C)	Reference(s)
tet(A)	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	210	60	Ng *et al.*, 2001
tet(O)	F: ACG GAR AGT TTA TTG TAT ACC R: TGG CGT ATC TAT AAT GTT GAC	171	57	Aminov *et al.*, 2002
tet(X)	F: AGC CTT ACC AAT GGG TGT AAA R: TTC TTA CCT TGG ACA TCC CG	278	60	Ghosh *et al*., 2009
erm(B)	F: GAT ACC GTT TAC GAA ATT GG R: GAA TCG AGA CTT GAG TGT GC	364	58	Chen *et al.* 2007
erm(T)	F: CAT ATA AAT GAA ATT TTG AG R: ACG ATT TGT ATT TAG CAA CC	369	51	Chen *et al.* 2007
sul1	F: CCG TTG GCC TTC CTG TAA AG R: TTG CCG ATC GCG TGA AGT	67	60	Heuer and Smalla, 2007
16S rRNA	F: CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	202	60	Muyzer *et al.*, 1993

Real-time PCR will be conducted using an Eppendorf Mastercycler *realplex* thermal cycler (Westbury, NY). Each gene will be quantified in duplicate from each of the triplicate genomic DNA extractions. PCR conditions and primer concentrations will be optimized to eliminate the formation of primer-dimers and non-specific products using a dissociation curve (data not shown; we have successfully completed this optimization for many of the proposed qPCR protocols). A typical PCR run will consist of a 10 min initial denaturation at 95°C, followed by forty cycles of denaturation at 95°C for 15 s and anneal/extension for 1 min at a temperature specific for the target gene. A typical 20 µL reaction mixture will contain 10 µL of 2× Power SYBR Green Master Mix (Applied Biosystems), 20 µg bovine serum albumin, optimized quantities of forward and reverse primers and ~1 ng of template DNA.

Standards for quantitative PCR will be prepared by PCR amplification of genes from positive control strains, followed by ligation into pGEM-T Easy vectors following manufacturer's instructions (Promega; Madison, WI), and transformation into E. *coli* DH5α. Plasmids will be purified using the alkaline lysis procedure (Sambrook *et al*., 1989). Plasmid DNA will be quantified by staining with Hoechst 33258 dye and measured on a TD-700 fluorometer (Turner Designs; Sunnyvale, CA) using calf thymus as a DNA standard. Ten-fold serial dilutions of plasmid DNA will be prepared and run on the thermal cycler to generate standard curves.

The LaPara laboratory has substantial experience with quantitative real-time PCR, including many of the protocols described herein. The techniques, once optimized, are extremely high-throughput such that a single person can perform several thousand qPCR reactions in a week. The majority of effort for these experiments, in fact, will focus on sample collection and statistical analyses. These statistical analyses will include numerous multivariate statistical techniques (e.g., non-metric multidimensional scaling (nMDS) and correspondence analysis) that will attempt to identify statistically significant correlations between gene quantities, antibiotic concentrations, and land use patterns (i.e., agricultural vs. urban, etc) in analogous fashion to a study that analyzed antibiotic resistance gene occurrence in the Haihe River in China (Luo *et al.* 2010).

5. Results and Deliverables

The main deliverable from this project will be the quantification of free antibiotic chemicals and of antibiotic resistant bacteria along a gradient of urban and agricultural landscapes. This research, therefore, will clarify the relative importance of treated municipal wastewater versus agricultural runoff in a Minnesota watershed. Although research similar to this has been performed elsewhere in the United States, there are several pertinent characteristics make this research especially important. First, no existing studies have done such a comprehensive simultaneous survey of both antibiotic concentrations and antibiotic resistance levels at the same sites. Second, animal feeding operations in Minnesota are constrained with respect to their antibiotic use as well as the rates at which manure can be applied to soil (i.e., "factory farms" do not exist in Minnesota as in other states (Minnesota Pollution Control Agency, 2000)). The proposed research, therefore, will be of substantial importance as the State of Minnesota potentially considers initiatives to reduce the presence of antibiotics and antibiotic resistant bacteria in its surface waters.

6. Timetable

This is a two-year project, beginning in July 2011. The timetable for completion of the described project illustrates when each task will be performed. "I" designates activities associated with the initial sampling effort (Summer 2011), and "M" designates activities associated with the main sampling effort (Spring-Fall 2012). In addition to the research tasks included on the timeline all three main investigators will be involved in generating required reports throughout the project, dissemination of results at conferences or meetings, and preparation of manuscripts for publication of results in peer-reviewed publications.

	'11						'12											'13					
Task	J	A	S	O	N	D	J	F	M	A	M	J	A	S	O	N	D	J	F	M	A	M	J
1	I	I	I	I	I	I	I	I	M	M	M	M	M	M	M	M	M						
2	I	I	I	I	I	I	I			M	M	M	M	M	M	M	M	M	M	M	M	M	M
3		I	I	I	I	I	I	I	I	I					M	M	M	M	M	M	M	M	M

Task 1: **Collect samples and quantify culturable antibiotic-resistant organisms (Wammer)**

For the 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.

Task 2: Measure antibiotic concentrations (Stoll)

Samples from the initial sampling effort will be screened for the presence of the same four antibiotics listed in Task 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.

Task 3: Quantify antibiotic resistance genes (LaPara)

Sample collection and extraction/purification of genomic DNA will be a substantial portion of the effort to quantify genes. Once the samples have been collected and purified, quantitative PCR should proceed rapidly as real time PCR is extremely high-throughput. In our prior experience, an individual student was able to perform more than 400 quantifications per day. Statistical analysis of the data will also require a substantial effort, as we anticipate a relatively large dataset.

7. Budget

The budget is as outlined on the previously submitted proposal. See Attachment A.

8. Credentials

Kristine H. Wammer

Education
B.S., Chemistry, 1997, St. Olaf College, Northfield, MN.
Ph.D., Civil and Environmental Engineering, 2003, Princeton University, Princeton, NJ.

Employment
Post-doctoral Fellow, 2003-2005, Departments of Environmental Health Sciences, Chemistry, and Civil Engineering, University of Minnesota, Minneapolis, MN.
Assistant Professor, 2005-present, Department of Chemistry, University of St. Thomas, St. Paul, MN.

Research
Dr. Wammer's research focuses on elucidating the chemical and microbiological processes affecting fate of organic contaminants in the aquatic environment. Her group's current interests include the environmental photochemistry and potential biological impacts of certain classes of pharmaceutical and personal care products.

Selected Relevant Publications
Hu L, Stemig AM, Wammer KH, Strathmann TJ. Oxidation of antibiotics during water treatment with potassium permanganate: reaction pathways and deactivation. Submitted to *Environmental Science and Technology*, 2010.

Werner JJ, Chintapalli M, Lundeen RA, Wammer KH, Arnold WA, McNeill K. 2007. Environmental photochemistry of tylosin: efficient, reversible photoisomerization to a less-active isomer, followed by photolysis. *Journal of Agricultural and Food Chemistry* **55**:7062-7068.

Wammer KH, LaPara TM, McNeill K, Arnold WA, Swackhamer DL. 2006. Changes in antibacterial activity of triclosan and sulfa drugs due to photochemical transformations. *Environmental Toxicology and Chemistry* **25**:1480-1486.

Timothy M. LaPara

Education
B.S.C.E., Civil Engineering, 1995, University of Notre Dame, Notre Dame, IN.
Ph.D., Environmental Engineering, 1999, Purdue University,

Employment
Post-doctoral Research Associate, 2000, Department of Biological Sciences, Purdue University, West Lafayette, IN.
Assistant Professor, 2000-2006, and Associate Professor, 2006-present, Department of Civil

Engineering, University of Minnesota, Minneapolis, MN.

Research
Dr. LaPara's research is focused on the role of municipal and industrial wastewater treatment plants in preserving environmental quality and in protecting public health. His research has a strong interdisciplinary nature, stemming from his unique background in both environmental engineering and microbiology.

Selected Relevant Publications
Ghosh S, Ramsden SJ, LaPara TM. 2009. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Applied Microbiology and Biotechnology* **84**:791-796.

Ghosh S, Sadowsky MJ, Roberts MC, Gralnick JA, LaPara TM. 2009. *Sphingobacterium* sp. strain PM2-P1-29 harbours a functional *tet*(X) gene encoding for the degradation of tetracycline. *Journal of Applied Microbiology* **106**:1336-1342.

Ghosh S, LaPara TM. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME Journal* **1**:191-203.

Dwight R. Stoll

Education
B.S., Plant Biology, 1999, and B.S., Biochemistry, 2001, Minnesota State University, Mankato, Mankato, MN.
Ph.D., Analytical Chemistry, 2007, University of Minnesota, Minneapolis, MN.

Employment
Instructor, 2005-2006, Department of Chemistry, St. Olaf College, Northfield, MN.
Post-doctoral Fellow, 2007-2008, Departments of Biochemistry, Molecular Biology, and Biophysics, and Medicine, University of Minnesota, Minneapolis, MN.
Assistant Professor, 2008-present, Department of Chemistry, Gustavus Adolphus College, St. Peter, MN.

Research
Dr. Stoll's research is focused on the development of novel separations based methods for the determination of trace level compounds in complex matrices such as environmental and biological samples. He uses multidimensional separations coupled with detection methods that include mass spectrometry and UV absorbance spectroscopy.

Selected Relevant Publications
Simpkins, SS, Bedard JW, Groskreutz SR, Swenson MM, Liskutin TE, Stoll, DR. 2010. Targeted three-dimensional liquid chromatography - A versatile tool for quantitative trace analysis in complex matrices. *Journal of Chromatography, A*, In Press.

Stoll DR. 2010. Recent progress in online, comprehensive two-dimensional high-performance liquid chromatography for non-proteomic applications. *Analytical and Bioanalytical Chemistry*, **397**:979-86.

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9. Dissemination and Use

Results from this project will be disseminated through scholarly publications in peer-reviewed journals. The undergraduate students involved in this project will also present results at regional conferences such as the *Minnesota Water* conference and will apply for funding to present results at national meetings such as the *American Chemical Society* meeting. In addition, we have made appropriate individuals at state and local agencies and organizations aware of this project and will maintain dialog with them as we obtain results. Specifically, we have contacted people at MDH, MDA, MPCA, and the Minnesota River Board. Finally, we will stay in close contact with another LCCMR-funded group doing complementary work that is primarily focused on endocrine disrupting compounds in the Zumbro River (D. Swackhamer, PI). We will inform each other of our findings in a timely fashion and potentially adapt accordingly.

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