2014 Project Abstract
For the Period Ending June 30, 2017

PROJECT TITLE: Antibiotics and antibiotic resistance genes in Minnesota lakes
PROJECT MANAGER: William Arnold
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2014, Chp. 226, Sec. 2, Subd. 03e

APPROPRIATION AMOUNT: $300,000
AMOUNT SPENT: $300,000
AMOUNT REMAINING: $0

Overall Project Outcomes and Results

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

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

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

The abundance of 48 antibiotic, metal, and antibiotic-associated resistance genes were quantified in the sediment cores with detected levels ranging from 10^3 to 10^8 gene copies per gram. Most ARGs included in this study, however, were not consistently quantifiable throughout the sediment cores.

Similar concentrations of *bla*$_{SHV}$, *cadA, copA, intI1*, and *mexB* were measured amongst the sediment cores, but Lake Winona had higher levels of *sul3* and *tet*(A) compared to the other lakes. ARGs levels did not appear to be a function of sediment core depth, and thus the measured levels are at or close to natural, indigenous background levels of the studied genes. Also, (unlike the antibiotics studied) ARG abundance did not appear to be a function of agricultural activity or degree of wastewater impact. Therefore, ARG abundance in the studied lakes is likely not influenced by antibiotic usage, but rather may be influenced by the presence of heavy metals that are known to co-select for ARGs.

Project Results Use and Dissemination

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

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

Date of Report: August 21, 2017

Date of Next Status Update Report:

Date of Work Plan Approval: June 4, 2014

Project Completion Date: June 30, 2017

Does this submission include an amendment request? Yes

PROJECT TITLE: Antibiotics and antibiotic resistance genes in Minnesota lakes

Project Manager: William Arnold

Organization: University of Minnesota

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Location: Statewide

Total ENRTF Project Budget: $300,000	
ENRTF Appropriation:	**$300,000**
Amount Spent:	**$300,00**
Balance:	**$0**

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

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

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

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

III. PROJECT STATUS UPDATES:

Project Status as of January 1, 2015:
Sediments from Lake Pepin, Lake Winona, Little Lake Wilson, and Duluth Harbor have been collected. Water and organic content have been determined for all of the sites. Dating for Lake Pepin has been completed and is underway for the other three cores. A suite of antibiotics have been purchased and verification of chromatography and extraction methods is in progress. DNA has been extracted from the Lake Pepin core and is ready for analysis.

Project Status as of July 1, 2015:
DNA has been extracted from sediment samples from all 4 lake cores and 16S rRNA and *intI1* qPCR have been performed on all the samples. Water content has been removed from various sediments subsamples via freeze drying. A liquid chromatography tandem mass spectrometry method has been developed that successfully separates the investigated antibiotics. Method development for the sediment extraction is currently in progress. The sediment cores collected during the 2014 summer have been dated using lead-210 methodology and magnetic susceptibility by Dan Engstrom.

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

Amendment Request (01/12/2016)
Because personnel costs were higher than expected and travel costs lower than expected in Activity 1, $2,398 is shifted from travel to personnel.
Amendment Approved: 1/14/2016

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

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

Amendment Request (01/18/2017)
As data was collected on gene and antibiotic levels, it was realized that the analyses for genes needed to be re-run to improve detection limits and that the extraction/analytical protocols for tetracyclines needed to be improved. Additionally, other samples for antibiotics needed to be re-extracted and analyzed to improve data quality. This led to over spending on the supplies and instrument analytical time. Additional personnel support was obtained in the form of a student fellowship, so it is requested to move $5,500 from personnel in Activity 2 and $22,500 from personnel in Activity to 3 to supplies/instrument time ($18,000 in Activity 2 and $10,000 in Activity 3). These shifts provide sufficient funds to cover expenses already incurred and to complete the analyses of antibiotic concentrations before the project completion date.
Amendment Approved: [01/20/2017]

Overall Project Outcomes and Results:
Antibiotics are substances that stop the growth of or kill bacteria. Animal agriculture and human medicine are the largest consumers of antibiotics worldwide. A fraction of the antibiotic administered is excreted in its original form through urine and/or feces. These residues reach aquatic environments through the discharge of wastewater effluent or drainage and surface runoff from agricultural fields to which manure has been applied. The presence of antibiotics in the environment are of concern, because these chemicals may select for and proliferate the occurrence of antibiotic resistance genes (ARGs). ARGs allow bacteria to survive in the presence of an antibiotic. Heavy metals are also known to co-select for ARGs. The World Health Organization has identified antibiotic resistance as one of the major threats to global health. The increase in the prevalence of antibiotic resistant infections, coupled with the decrease in the development of new antibiotics, emphasize the need for new strategies to better understand antibiotic resistance. The goal of the project is to quantify the current and historical levels of selected human and veterinary antibiotic compounds and genes that code for their resistance in lake sediments. Sediment cores collected for three anthropogenically-impacted Minnesota lakes (Lake Pepin, Duluth Harbor, and Lake Winona) and a control lake in Superior National Forest (Little Wilson Lake) were radiometrically dated. The twenty antibiotics included in this study have a mixture of human and/or agricultural uses, some are known natural products, and they span several of the major classifications (sulfonamides, fluoroquinolones, tetracyclines, macrolides). Sediment cores were successful at capturing the usage trends of ten antibiotics. The initial appearance of antibiotics in the sediment core generally agreed with the FDA approval date, which provided further confidence in the dating of the sediment cores and the ability of sediment cores to capture antibiotic usage trends. Ofloxacin, trimethoprim, sulfapyridine, and sulfamethazine were the only antibiotics to be detected in all three anthropogenically-impacted studied lakes with levels up to 91.7, 2.5, 13.1, and 5 ng g^{-1}, respectively. Human-use antibiotics were detected more frequently and at higher concentrations than antibiotics used for veterinary medicine. Also, the degree of antibiotic pollution appeared to be a function of treated wastewater impact. Lake Winona was the most heavily wastewater impacted lake in the study (approximately 63% of the inflow is treated wastewater effluent) and had the highest concentrations and greatest number of antibiotics detected. Treated municipal wastewater is likely the primary contributor to antibiotic pollution in the studied lakes. The abundance of 48 antibiotic, metal, and antibiotic-associated resistance genes were quantified in the sediment cores with detected levels ranging from 10^3 to 10^8 gene copies per gram. Most ARGs included in this study, however, were not consistently quantifiable throughout the sediment cores. Similar concentrations of *bla*$_{SHV}$, *cadA, copA, intI1*, and *mexB* were measured amongst the sediment cores, but Lake Winona had higher levels of *sul3* and *tet*(A) compared to the other lakes. ARGs levels did not appear to be a function of sediment core depth, and thus the measured levels are at or close to natural, indigenous background levels of the studied genes. Also, (unlike the antibiotics studied) ARG abundance did not appear to be a function of agricultural activity or degree of wastewater impact. Therefore, ARG abundance in

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

Amendment Request (08/21/2017)
To complete the required analyses, unused travel funds from activity 1 (~$1,300) were used to conduct the final analyses of antibiotic concentrations of activity 2.

IV. PROJECT ACTIVITIES AND OUTCOMES:

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

Cores will be collected by a piston or box-type corer. Riverine surface sediment samples will be collected with a dredge or scoop, depending on the depth. The cores will be extruded in the field in 1 to 4 cm sections with subsamples being taken for dating and determination of resistance gene levels. The remainder of the sample will be dedicated to chemical analyses. The Lake Pepin core will be dated via magnetic susceptibility, and it will be sectioned in the laboratory after dating is performed. The other cores will be dated using lead-210 and cesium-137 methods and other chemical markers as described in Dr. Engstrom's recent work. We will collect cores that are deep enough (i.e., go back it time far enough) such that we will have core sections that date to prior to the deployment of the antibiotic classes (1930-1960 depending on the class). The water and organic matter content will be determined as a function of depth via loss on ignition analysis. Because antibiotic resistance levels may be related to heavy metal content, all sediment samples will be analyzed via inductively coupled plasma-mass spectrometry (ICP-MS; Department of Earth Sciences, U of MN) to determine the metal concentrations. Sediment deposition rates as a function of time will be calculated based on the mass of sediment contained between dated points in the core section.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 45.259
Amount Spent: $ 45,259
Balance: $ 0

Activity Completion Date:

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

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

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

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

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

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

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

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

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

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

ACTIVITY 2:

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

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

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

spectrometry (LC-MS/MS) with electrospray ionization (available in the U of MN Cancer Center on an hourly basis). From the data derived from analyses above, the concentrations (mass per mass) and accumulation rates (mass per area per time) of the antibiotics will be calculated. Because clinical use of antibiotics began in the 1930s, sediments deposited prior to this date will serve to reveal and natural background concentrations for those compounds that can be produced naturally (i.e., macrolides and tetracyclines).

Summary Budget Information for Activity 2:

ENRTF Budget: $ 140,741
Amount Spent: $ 140,741
Balance: $ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Optimize antibiotic extraction and analytical methods	7/31/15	$ 43,500
2. Measure antibiotic concentrations in sediment samples	12/31/16	$ 87,241
3. Calculate accumulation rates	1/31/17	$ 10,000

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

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

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

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

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

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

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

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

ACTIVITY 3: Measurement of antibiotic resistance as a function of depth/time in sediment cores

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

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

Summary Budget Information for Activity 3:

ENRTF Budget: $ 114,000
Amount Spent: $ 114,000
Balance: $ 0

Activity Completion Date:

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

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

Activity Status as of July 1, 2015:
DNA has been extracted from sediment samples from all 4 lake cores and 16S rRNA and *intI1* qPCR have been performed on all the samples.

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

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

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

Final Report Summary: All antibiotic resistance gene (ARG) analyses of the sediment core samples have been completed. Protocols to quantify 48 genes were developed. Concentrations of antibiotic and metal resistance genes ranged from 10^3 to 10^8 gene copies per gram of sediment. Several ARGs were generally not quantifiable in the sediment cores. A few ARGs (*bla*$_{SHV}$, *cadA, copA, intI1*, and *mexB*) had similar concentrations throughout the sediment cores. Lake Winona had noticeably higher levels of *sul3* and *tet*(A). This study saw no correlation between the levels of agricultural activity and degree of treated municipal wastewater and concentrations of ARGs in lake sediments. The abundance of ARGs did not appear to a function of sediment core depth, thus the quantities measured are at or close to natural, indigenous background levels of these genes. Alternatively, the presence of heavy metals within the sediments were found to strongly correlate to ARG levels.

V. DISSEMINATION:

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

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

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

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

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

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

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

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Explanation
Personnel:	$ 250,000	Arnold at 4-6% time per year. LaPara at 1-2% time per year. Graduate students (43-50% time) and/or postdoc (75% time). Costs include fringe benefits for all and tuition for the graduate student.
Professional/Technical/Service Contracts:	$ 16,000	Science Museum of Minnesota and Daniel Engstrom for assistance with core collection and dating.
Equipment/Tools/Supplies:	$ 29,000	Chemical standards, isotope standards, microbiologial/DNA extraction kits, instrument/analytical time for antibiotic and DNA analysis, solvents, consumable supplies, notebooks, software licenses. Equipment maintenance.
Travel Expenses in MN:	$ 5,000	Mileage charges and university vehicle rental charges for trips to collect water samples. Hotel/meal charges if overnight stay required.
TOTAL ENRTF BUDGET:	**$ 300,000**	

Explanation of Use of Classified Staff: not applicable

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

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

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

B. Other Funds:

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

VII. PROJECT STRATEGY:

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

B. Project Impact and Long-term Strategy:

This project will provide an understanding of the historical levels of antibiotics used in human and veterinary medicine that have entered Minnesota lakes. Additionally, this will be the first study to investigate how the discharge of these chemicals has or is affecting the levels of resistance genes in the environment. This is information critical to protecting human and ecological health and may provide information relevant to antibiotic use and development. This study will reveal if additional treatment to remove antibiotics from wastewater or runoff is necessary or unnecessary in terms of proliferation of resistance genes.

VIII. ACQUISITION/RESTORATION LIST: not applicable

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

X. ACQUISITION/RESTORATION REQUIREMENTS WORKSHEET: not applicable

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

XII. REPORTING REQUIREMENTS:

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

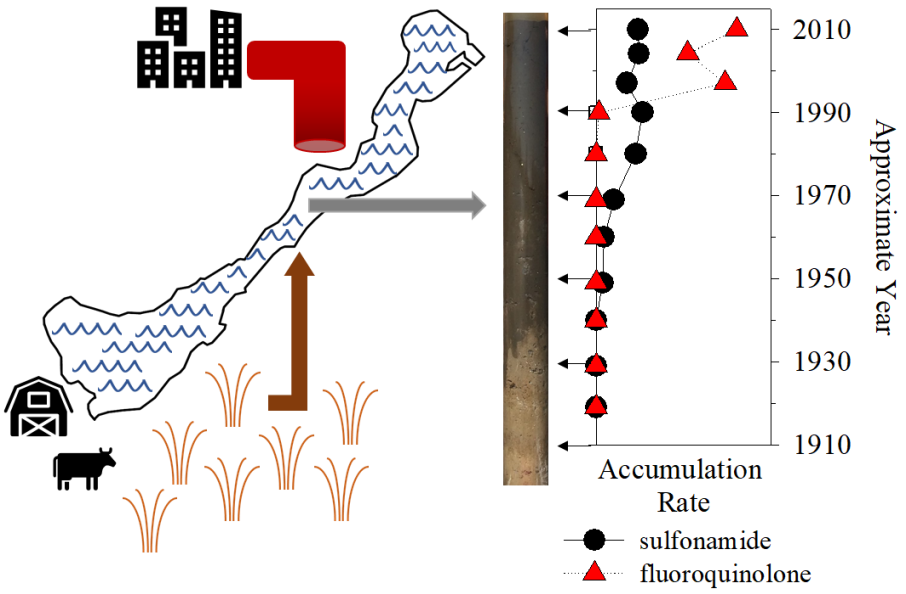
Environment and Natural Resources Trust Fund
M.L. 2014 Project Budget

Project Title: Antibiotics and antibiotic resistance genes in
Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03e
Project Manager: William Arnold
Organization: University of Minnesota
M.L. 2014 ENRTF Appropriation: $ 300,000
Project Length and Completion Date: 3 Years, June 30, 2017
Date of Report: August 4, 2017

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Revised Activity 1 Budget 8/4/17	Amount Spent	Activity 1 Balance	Activity 2 Budget	Revised Activity 2 Budget 8/4/17	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM													
Personnel (Wages and Benefits)	$26,898	$26,898	$26,898	$0	$107,500	$107,500	$107,500	$0	$90,000	$90,000	$0	$224,398	$0
Arnold (PI, 6% time per year Y1 and Y2, 4% Y3. Estimated total: $36,200). Project supervision, supervision of graduate student #2/postdoctoral researcher #1 and project reporting.													
LaPara (co-PI, 2% time per year Y1 and Y2, 1% Y3. Estimated total: $9,600). Project supervision, supervision of graduate student #1 and and project reporting.													
Graduate student #1 (43.75-50% time in Y1 and Y2, 25-50% time in Y3. Estimated total: $122,700). Extraction and purification of DNA from collected sediment samples. Quantificaiton of resistance genes.													
Graduate student #2 (43.75-50% time in Y1 and Y2, 25-50% time in Y3. Estimated total: $81,500) or Postdoctoral Researcher #1 (75% time in Y1 and Y2). Sediment core collection and sectioning. Development of antibiotic extraction and analytical protocols. Determination of antibiotic concentrations in sediments.													
Professional/Technical/Service Contracts													
Science Museum of Minnesota for collection and dating of sediment cores. Costs include personnel (Dr. Daniel Engstrom, 2% effort $4688 salary, $1312 fringe) and analytical and dating costs ($10,000).	$16,000	$16,000	$16,000	$0								$16,000	$0
Equipment/Tools/Supplies													
Supplies including chemical standards, isotope standards, microbiologial/DNA extraction kits, instrument/analytical time for antibiotic and DNA analysis, solvents, consumable supplies, notebooks, software licenses	$1,000	$1,000	$1,000	$0	~~$30,000~~	$31,241	$31,241	$0	$22,000	$22,000	$0	$54,241	$0
Maintenance and repair of laboratory equipment required for analyses and experiments					$2,000	$2,000	$2,000	$0	$2,000	$2,000	$0	$4,000	$0
Travel expenses in Minnesota													
Mileage charges and univeristy vehicle rental charges for trips to collect water samples. Hotel/meal charges if overnight stay required.	~~$2,602~~	$1,361	$1,361	$0								$1,361	$0
COLUMN TOTAL	**$46,500**	**$45,259**	**$45,259**	**$0**	**$139,500**	**$140,741**	**$140,741**	**$0**	**$114,000**	**$114,000**	**$0**	**$300,000**	**$0**

Chapter 3: Sedimentary Record of Antibiotic Accumulation in Minnesota Lakes

This manuscript has been submitted to the journal *Environmental Science and Technology*.



3.1 Abstract

The widespread detection of antibiotics in the environment is concerning because antibiotics are designed to be effective at small doses. The objective of this work was to quantify the accumulation rates of antibiotics used by humans and animals, spanning several major antibiotic classes (sulfonamides, tetracyclines, fluoroquinolones, and macrolides), in Minnesota lake-sediment cores. Our goal was to determine temporal trends, the major anthropogenic source to lacustrine systems, and the importance of natural production. A historical record of usage trends for ten human and/or animal-use antibiotics (four sulfonamides, three fluoroquinolones, one macrolide, trimethoprim, and lincomycin) was faithfully captured in the sediment cores. Ten other antibiotics were not detected. Ofloxacin, trimethoprim, sulfapyridine, and sulfamethazine were detected in all of the anthropogenically-impacted studied lakes with maximum fluxes reaching 20.5, 1.2, 3.3, and 1.0 ng cm^{-2} yr^{-1}, respectively. Natural production of lincomycin may have occurred in one lake at fluxes ranging from 0.4 to 1.8 ng cm^{-2} yr^{-1}. Wastewater effluent appears to be the primary source of antibiotics in the studied lakes, with lesser inputs from agricultural activities.

3.2 Introduction

The health care system was revolutionized with the discovery of antibiotics in the 1930s. The ability to treat and prevent microbial infections resulted in antibiotics being one of the greatest inventions of the 20th century. The effectiveness of antibiotics has led to their mass production and widespread use. In 2011 and 2012, an estimated 17,900 tons of antibacterials were sold and distributed by retail and non-retail channels in the United States for use in humans and animals.[114–116] Given the large quantities of antibiotics used, it is important to understand their potential impact in aquatic systems.

Only a fraction of the antibiotics administered is metabolized by humans and animals; up to 90% of the dose is excreted in urine and feces.[188] Wastewater treatment plant (WWTP) effluents are point sources of human-use antibiotics to aquatic systems due to incomplete removal by conventional treatment technologies.[50,118] Concentrations of antibiotics in municipal wastewater are typically in the low µg/L range, and receiving water levels range from low to high ng/L.[1,2,4,122,123,125,127,128] The agriculture industry uses antibiotics to treat and prevent microbial illnesses and as growth promoters in livestock. Agricultural practices contribute to antibiotic pollution in water bodies by surface runoff from fields to which manure contaminated by antibiotics is applied.[123,127,129]

Antibiotics have also been detected downstream of wastewater outfalls in sediment.[4,122–126] Compounds, such as tetracyclines and fluoroquinolones, that strongly adsorb onto particles, accumulate in sediment.[189,190] Similar to surface water, the highest observed levels of antibiotics in sediments were downstream of metropolitan (industrial and municipal wastes) and agricultural and aquaculture areas (feedlots and fish ponds).[123–125] In addition to their mass production, some antibiotics are naturally

produced in the environment, such as select tetracyclines, penicillin, erythromycin, tylosin, and lincomycin.[191,192]

Different from other classes of contaminants of emerging concern, antibiotics are designed to have an effect on microorganisms.[4] Ecosystem health may be influenced by antibiotics by hindering the growth of algae and benthic invertebrates.[125,141,142] Use of antibiotics may increase the occurrence of antibiotic resistance in bacteria, which poses a risk to human and veterinary health by reducing the ability of antibiotics to treat microbial illnesses.[3,147] Lethal concentrations cause a specific immediate response, but prolonged sub-inhibitory levels of antibiotics can also select for and promote the dispersion of antibiotic resistant genes (ARGs).[147,148] Recent studies have shown conflicting findings as to whether environmental samples show correlations between antibiotic and ARG levels.[25,158,193,194]

The objective of this work is to quantify the current and historical levels of selected human- and animal-use antibiotics in lake sediment cores. Measuring levels of antibiotics in dated sediments is a useful tool to reconstruct chemical pollution of water bodies over time. Antibiotic levels in surface waters and surface sediments due to anthropogenic inputs have been well studied.[123,124,127,129] This study, however, aims to assess the trends of environmental levels of antibiotics throughout the past century due to anthropogenic inputs. Also investigated is whether synthetic or naturally produced antibiotics are more persistent in the environment, what the dominant source of antibiotic pollution is in the targeted lakes, and whether the degree of anthropogenic impact is reflected in the historical trends. The potential pressure of antibiotics selecting for ARGs provides motivation for further understanding in the abundance and persistence of

antibiotics in the environment. Thus, historical levels of 20 antibiotics (including those from the fluoroquinolone, tetracycline, sulfonamides, and macrolide classifications) were quantified in sediment cores from four Minnesota lakes.

3.3 Materials and Methods

Sediment Core Collection. Sediment cores were collected in August and September 2014 from four Minnesota lakes (Figure C.1). Lake Pepin (GPS coordinates: 44.499750, -92.294170) and the Duluth Harbor of Lake Superior (46.732783, -92.065333) were selected because they have large watersheds and receive multiple waste inputs. Lake Winona (45.87501, -95.40402) has a small watershed with one municipal wastewater discharge, and Little Wilson Lake in the Superior National Forest (47.656138, -91.067905) lacked any major waste inputs and served as a control site.

The cores were collected via a piston corer equipped with a polycarbonate tube and deployed into the sediment from the surface using Mg-alloy rods. The sediment cores were extruded vertically top-down and sectioned on site into 2 – 4 cm intervals, except for Lake Pepin. The outer circumference of the core was removed to prevent carryover of younger to older sediment via smearing during extrusion. Sections were stored in cleaned glass jars, homogenized, and a subsample was taken for radiometric dating. Samples were cooled to 4 °C in the field and were subsequently kept at -20 °C for long term storage. Because a magnetic susceptibility profile of Lake Pepin was used to determine the deposition date of core sections (see below), this core was sectioned in the laboratory.

Loss-on-ignition tests on homogenized samples were used to determine water, organic carbon, carbonate, and inorganic content of sediment by weighing the sediment after heating for 12 hours at 105 °C, 4 hours at 550 °C, and 2 hours at 1000 °C, respectively. An aliquot of sediment (approx. 10 g dry wet) at select intervals was freeze-dried and then stored at -20 °C until extraction for antibiotic analysis.

The antibiotics selected for this study include six sulfonamides (sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, and sulfapyridine), three macrolides (erythromycin, roxithromycin, and tylosin), four tetracyclines (chlortetracycline, doxycycline, oxytetracycline, and tetracycline), four fluoroquinolones (ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin), and three non-categorized antibiotics (carbadox, lincomycin, and trimethoprim). The β-lactams amoxicillin, penicillin G, and penicillin V were originally included in the analysis suite, but degraded during the extraction process. β-lactams are known to undergo hydrolysis readily[195] and are infrequently detected in surface waters and wastewater effluents.[4,196]

Chemicals sources and purities are in Supporting Information (SI). The antibiotics chosen for this study include those that: 1) are natural products; 2) had human and/or animal uses; 3) were part of several major classifications; and 4) had been previously detected in sediment samples. The compounds, their abbreviations, and uses are given in Table 3.1. Degradation products of chlortetracycline (epi-chlortetracycline, iso-chlortetracycline, and epi-iso-chlortetracycline) and erythromycin (erythromycin-H_2O) were also included and summed into their respective parent compound concentrations.

Table 3.1. List of antibiotics included in the study separated into classifications with their respective abbreviations and general uses. Also noted is whether antibiotic is naturally produced and if it is on the World's Health Organization 19th list of essential medications.[106]

Antibiotic	Acronym	Natural Production	General Uses[197]	List of Essential Medications
		Sulfonamides		
sulfachlorpyridazine	SCP	no	swine, calves, dogs	no
sulfadiazine	SDZ	no	horses, humans	yes
sulfadimethoxine	SDM	no	fish, poultry	no
sulfamethazine	SMZ	no	swine, cattle	no
sulfamethoxazole	SMX	no	human	yes
sulfapyridine	SPD	no	human	no
		Macrolides		
erythromycin	EMC	yes	humans, poultry, swine	yes
roxithromycin	RXC	no	humans	no
tylosin	TYL	yes	chicken, swine, cattle	no
		Tetracyclines		
chlortetracycline	CTC	yes	swine, poultry, cattle, sheep, ducks	no
doxycycline	DXC	no	human, dogs	yes
oxyetracycline	OTC	yes	poultry, fish, swine, cattle, sheep	no
tetracycline	TCC	yes	human, dogs, cattle	yes
		Fluoroquinolones		
ciprofloxacin	CFC	no	human, swine, chickens	yes
enrofloxacin	EFC	no	cattle, swine, poultry, dogs, cats	no
norfloxacin	NFC	no	human, poultry	no
ofloxacin	OFC	no	poultry, human	yes
		Non-Categorized		
carbadox	CBX	no	swine	no
trimethoprim	TMP	no	human, dogs, horses	yes
lincomycin	LMC	yes	poultry, swine	no

Radiometric Dating. Sediment cores were dated by lead-210 (^{210}Pb) methods, as described previously.[44–46] Briefly, ^{210}Pb was quantified by alpha spectrometry of its daughter isotope polonium (^{210}Po), with dates and sediment accumulation rates calculated according to the constant rate of supply model.[45,46] Core-specific rates of sediment accumulation were corrected for sediment focusing based on the inventory of ^{210}Pb in the core to derive mean whole-lake accumulation rates (see Anger et al[44] SI for details).

Analyte concentrations were converted to accumulation rates (fluxes) by multiplying by the focus-corrected sediment accumulation rate for each analyzed interval. The Lake Pepin core was dated by matching the magnetic profile to that of cores collected previously.[44]

Extraction and Analysis. Proper analytical cleaning procedures were followed to prevent sample contamination. Details are in the SI. Internal standards (clinafloxacin, $^{13}C_2$-erythromycin, $^{13}C_2$-erythromycin-H_2O, simeton, and $^{13}C_6$-sulfamethoxazole, 100 ng) and surrogates (demeclocycline, nalidixic acid, and $^{13}C_6$-sulfamethazine, 20 ng) were spiked onto sediment in a methanol solution prior to extraction. Two sediment extraction methods were used: accelerated solvent extraction (ASE) and ultrasound assisted extraction (UAE). The following is a brief description of both extraction methods with a more detailed description provided in the SI. The ASE method was optimized to extract antibiotics from 0.5 or 1 g of sediment with 50:50 methanol:50mM pH 7 phosphate buffer at 100 °C, heated for 5 min followed by 2 cycles of 5 min static periods. Less sediment was used for samples with higher organic content (Lake Winona and Little Wilson Lake) to facilitate the clean up using solid phase extraction (SPE). The UAE method was adapted from Wallace and Aga.[198] Sediment (0.5 g) was mixed with Ottawa sand (2.5 g) and suspended in 10 mL of 20:30:50 acetonitrile:methanol: 0.1 M ethylenediaminetetraacetic acid (EDTA)/0.08 M disodium phosphate/0.06 M citrate buffer (pH 4) solution, vortexed (30 sec), placed in an ultrasound bath (40 kHz, 10 min), and centrifuged (3300 rpm, 10 min). The UAE was repeated two additional times per sample, and extracts were combined.

Organic solvents were removed from sediment extracts using a rotary evaporator in a 35 °C water bath. ASE aqueous extracts were spiked with 250 µL of 20:80 formic acid: 10% sodium chloride/0.5% EDTA solution. The Little Wilson Lake ASE extracts were diluted to 500 mL with ultrapure water before loaded onto the solid phase extraction (SPE) cartridge due to higher organic content. UAE aqueous extracts were diluted to 400 mL and adjusted to pH 4 with phosphoric acid.

An SPE method adapted from Meyer et al.[197] was used to remove interferences from the extracts and concentrate the sample. Two different sorbents were used for SPE, Oasis HLB (6cc, 200 mg, 30 µm) and Oasis MCX (6 cc, 150 mg, 30 µm) cartridges. Both cartridges were cleaned with 10 mL of methanol and ultrapure water. Samples were loaded in tandem with HLB on top of MCX under vacuum that did not exceed 15 mm Hg. Cartridges were then disassembled and the HLB was washed with 40:60 methanol:water (6 mL) and MCX with water (3 mL). Cartridges were eluted with MCX on top of HLB. Methanol (3 mL) was added to HLB prior to placing MCX on top. Methanol (5 mL, ×2) was then added to MCX and eluted through both cartridges. MCX was also eluted separately with 3 mL of 5% ammonium acetate in methanol that was combined with the methanol eluent. Cartridges were eluted on the manifold into 15-mL centrifuge tubes. Vacuum pressure was used to start the elution, then subsequently eluted by gravity. Eluents were blown down to dryness with industrial grade nitrogen in a 40 °C water bath. Samples were resuspended in 20 mM ammonium acetate (200 µL) and any particles were removed with a syringe filter (GHP, 0.4 µm) prior to liquid chromatography tandem mass spectrometry analysis. Additional details are in the SI.

Several quality assurance and control measures were taken to assure the precision of reported antibiotic concentrations. One duplicate per core was extracted to monitor reproducibility. Extraction efficiency was monitored in triplicate from Ottawa sand and from each core with pre-1900s sediment. Method blanks were extracted at least every eight samples to monitor and correct for any carryover contamination. Method blanks were comprised of either Ottawa sand or pre-1900s sediment and were spiked with surrogates and internal standards and subjected to the entire extraction process.

ASE samples were analyzed on an Agilent 1100 high pressure liquid chromatograph (HPLC) equipped with a Thermo TSQ Vantage triple quadrupole tandem mass spectrometer (MS/MS) in positive electrospray ionization mode. Separation was performed on a Phenomenex Kinetex F5 (1.7 µm, 100 Å, 50 × 2.1 mm) column with a SecurityGuard ULTRA guard column. Flow rate was maintained at 250 µL/min, temperature was set to 50 °C, and 8 µL was injected onto column. The HPLC-MS/MS was shared among several researchers, so the system was flushed with a 50:50 10 mM EDTA:methanol solution for 30 minutes prior to each analysis to remove metals from the system and improve peak shapes of tetracyclines and fluoroquinolones. A gradient elution of mobile phases 0.1% formic acid in ultrapure water and 0.1% formic acid in acetonitrile was developed, see Table S3, and flow was diverted to waste from 0 to 1 and 7.5 to 25 min. Due to the number of analytes included in the study, each sample was analyzed by three HPLC-MS/MS methods that monitored for: (1) sulfonamides and surrogates; (2) tetracyclines and fluoroquinolones; and (3) others and macrolides.

A Thermo Dionex ultimate 3000 RSLCnano system replaced the Agilent 1100 HPLC prior to analysis of the UAE sediment extracts. A Waters XSelect CSH C18 (3.5

μm, 130 Å, 50 × 2.1 mm) column was used. Separation of antibiotics was achieved with a flow rate of 0.5 mL/min, 8 μL injection volume, and temperature at 35 °C. From 0 to 1.5 min and 5.5 to 20 min, flow was diverted to waste. Two gradient elution methods consisting of 0.1% formic acid in water and 0.1% formic acid in methanol were developed, see Table S4. UAE samples were also analyzed by three methods: (1) sulfonamides, $^{13}C_6$-sulfamethazine, and others; (2) tetracyclines, fluoroquinolones, demeclocycline, and nalidixic acid; and (3) macrolides.

Analytes were detected and quantified using single reaction monitoring (SRM) transitions, (Table S5). An additional SRM was monitored for each analyte to confirm the identity of quantified peak. The mass spectrometer sensitivity varied between analyses, and thus parameters were optimized with the infusion of 5μM simeton in 50:50 20 mM ammonium acetate:acetonitrile (or methanol for UAE analysis) prior to each analysis. Typical values for mass spectrometer parameters were: scan time 0.02 sec; scan width: 0.15; Q_1/Q_3: 0.7; spray voltage: 3300 V; sheath gas pressure: 18 psi; capillary temperature: 300 °C; collision pressure: 1.5 mTorr; declustering voltage: -9 V; and tube lens: 95.

Limits of quantification (LOQs) were calculated from 10× the peak area of an analyte's retention time in the method blank minus the mass calculated from the method blank. Limits of detection (LODs) were calculated from 3× the peak area in the method blank at the retention time of an analyte. Antibiotic accumulation rates above LOQ were calculated from recovery corrected sediment concentrations using isotope dilute methodology and were sediment focusing corrected to determine antibiotic accumulation

on a whole-lake scale, see SI for equations. Reported LOQs and LODs were recovery and sediment focus corrected for each lake.

3.4 Results

Loss-On-Ignition Results and Dating. Organic, carbonate, and inorganic content of Little Wilson Lake, Duluth Harbor, Lake Pepin, and Lake Winona sediment cores and percent water of sample determined by loss-on-ignition are in Figure C.2 and Tables C.6-C9. Little Wilson Lake had the highest organic content (39.0 ± 1.1%) followed by Lake Winona (19.4 ± 2.0%), Lake Pepin (12.3 ± 1.6%), and Duluth Harbor (10.7 ± 1.1%). Results from ^{210}Pb dating are similar to those for cores taken previously from the same lakes and core-sites (Table C.10-C.12).[32, 34] Mean dry-mass accumulation rates (DMAR) range from 0.04 g cm^{-2} yr^{-1} (Little Wilson) to 0.43 g cm^{-2} yr^{-1} (Lake Pepin), and increase by 5-7× from c. 1860 to present day in the Pepin, Winona, and Duluth Harbor cores. DMAR are relatively constant over time in Little Wilson.

Analytical Method Performance. Two liquid chromatography tandem mass spectrometry methods were developed. Both methods/stationary phases gave linear calibration curves ranging from 0.5 to 450 µg/L and were of good quality for all analytes ($R^2 > 0.95$). See Figures C.3 – C.7 for representative chromatograms.

LODs for antibiotics via ASE ranged from 0.06 to 3.74 ng/g for macrolides, 0.08 to 0.68 ng/g for sulfonamides, 0.5 to 22.6 ng/g for tetracyclines, 0.03 to 19.75 ng/g for fluoroquinolones, and 0.05 to 1.03 ng/g for non-categorized antibiotics. ASE produced LOQs that varied from 0.06 to 11.22 ng/g for macrolides, 0.28 to 2.03 ng/g for sulfonamides, 1.6 to 55.7 ng/g for tetracyclines, 0.08 to 59.24 ng/g for fluoroquinolones,

and 0.13 to 3.43 ng/g for non-categorized antibiotics. Table S13 contains specific antibiotic LOD and LOQ values. ASE extraction efficiencies varied among antibiotic classes and between cores. Relative recoveries varied from 85 to 277 % for macrolides, 70 to 224 % for sulfonamides, 1 to 122 % for tetracyclines, 3 to 102 % for fluoroquinolones, and 12 to 82% for non-categorized. It is important to note that, while low extraction efficiencies lead to an increase in uncertainty in measured antibiotic concentrations, the observed temporal trends should not have been affected. Absolute and relative recoveries for internal standards, surrogates, and antibiotics via the ASE method are in Table S14.

The UAE method produced LODs and LOQs ranges of 0.02 to 1.56 ng/g and 0.05 ng/g to 4.68 ng/g, respectively, with non-categorized antibiotics and sulfonamides generally having the lowest detection limits followed by fluoroquinolones and tetracyclines. Relative recoveries varied from 60 to 106 % for sulfonamides, 34 to 123 % for tetracyclines, 36 to 53% for fluoroquinolones, and 23 to 157 % for non-categorized compounds. Table S15 and Table S16 contain analyte specific absolute and relative recoveries and LODs and LOQs, respectively, for UAE extracts.

Antibiotics in Minnesota Lakes. The depth profiles of detected antibiotics in Lake Pepin, Lake Winona, and Duluth Harbor are shown in Figure 3.1 in terms of whole-lake (focusing corrected) accumulation rates (ng cm^{-2} yr^{-1}). Figure C.8 shows recovery corrected sediment concentrations (in ng antibiotic/g sediment) throughout sediment cores for the detected antibiotics. The reported accumulation rates in Figure 3.1 were determined using ASE, except for trimethoprim in Lake Winona which were determined by UAE. Contamination by trimethoprim during the ASE extraction of Lake Winona,

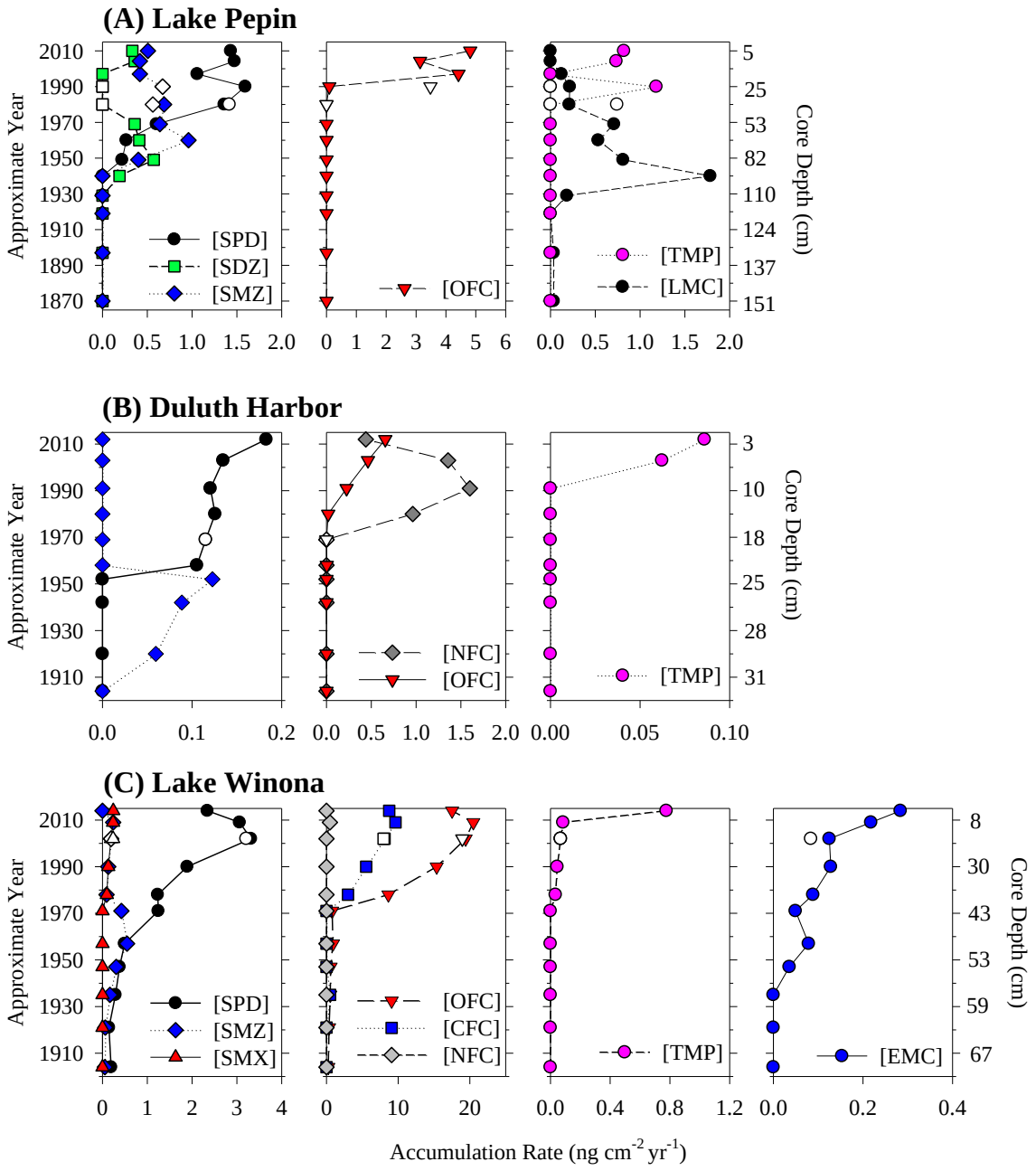


Figure 3.1. Focus-corrected accumulation rates (ng cm^{-2} yr^{-1}) of sulfapyridine (SPD), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfamethoxazole (SMX), ofloxacin (OFC), ciprofloxacin (CFC), norfloxacin (NFC), trimethoprim (TMP), lincomycin (LMC), and erythromycin (EMC) in sediment cores from: (A) Lake Pepin; (B) Duluth Harbor; and (C) Lake Winona. White symbols indicate replicates. Concentrations in ng/g are given in Figure C.8.

which was resolved when the UAE extraction was performed, resulted in no discernable trend. Other accumulation rates determined by UAE are not shown because they are highly similar to those determined by ASE, see Figure 3.2 and C.9. No antibiotics were measured in the control lake, Little Wilson Lake, except for single detection of ciprofloxacin and norfloxacin in 1916 and 1990 sediment, respectively. Their presence is likely due to carry over contamination during ASE extraction given their single occurrences.

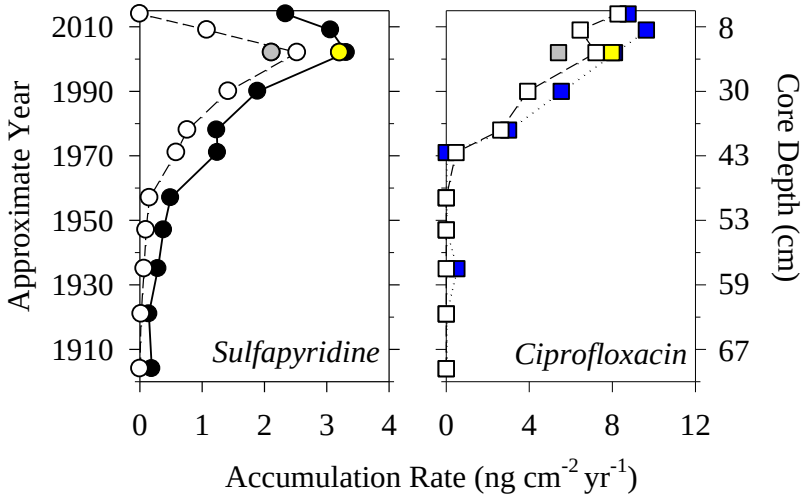


Figure 3.2. Focus-corrected accumulation rates (ng cm^{-2} yr^{-1}) of sulfapyridine and ciprofloxacin in Lake Winona. White symbols represent accumulation rates determined by ultrasound assisted extraction (UAE) method. Colored symbols are accumulation rates quantified by accelerated solvent extraction (ASE) method. Grey and yellow symbols are UAE and ASE replicates, respectively. Additional comparisons are in Figure C.9.

Sulfonamides. Sulfonamides are a group of antibiotics that were first synthesized in the late 1930s. Of the six sulfonamides included in this study, only sulfapyridine and sulfamethazine were detected in all three anthropogenically-impacted lakes. Sulfapyridine is a human-use antibiotic that received FDA approval in 1939, but marketing was discontinued in 1990. It was first detected in Lake Pepin and Duluth

Harbor ca. 1950. In Lake Winona, accumulation rates of sulfapyridine ranged from 0.2 to 0.5 ng cm^{-2} yr^{-1} prior to 1960, but increased to 1.2 ng cm^{-2} yr^{-1} around 1970. Sulfapyridine had the highest accumulation rates of the sulfonamides, with the highest fluxes observed in Lake Winona (3.3 ng cm^{-2} yr^{-1} ca. 2000) followed by Lake Pepin (1.6 ng cm^{-2} yr^{-1} in 1990) and then Duluth Harbor (0.18 ng cm^{-2} yr^{-1} in 2010). Sulfamethazine, which is used to promote growth and prevent diseases in animals, reached accumulation rates of 0.12, 0.96, and 0.54 ng cm^{-2} yr^{-1} in Duluth Harbor, Lake Pepin and Lake Winona, respectively. The first occurrence of sulfamethazine in Lake Pepin corresponded with its 1949 FDA approval. Sulfamethazine was present throughout the Lake Winona core, and appeared from 1920 to 1950 in Duluth Harbor.

Sulfadiazine is used for both human and agricultural treatments. After its first appeared in Lake Pepin near its 1941 FDA approval, fluxes varied from non-detect to 0.57 ng cm^{-2} yr^{-1} to the present day. A human-use only drug, sulfamethoxazole was only detected in Lake Winona. It first appeared about 1980 (corresponding to the year of FDA approval) at 0.09 ng cm^{-2} yr^{-1} and increased to a present-day flux of 0.24 ng cm^{-2} yr^{-1}. Two agricultural sulfa drugs, sulfachlorpyridazine and sulfadimethoxine, were not detected.

Fluoroquinolones. The only fluoroquinolone present in all three wastewater-impacted lakes was ofloxacin, a synthetic antibiotic used by poultry and humans. Ofloxacin levels generally increase near the 1990 FDA approval. In Lake Winona, ofloxacin was detected throughout the core, but accumulation rates increased dramatically in the 1980s from 0.8 to 8.6 ng cm^{-2} yr^{-1}, reaching a maximum flux of 20.5 ng cm^{-2} yr^{-1} in the 2010s before decreasing to 17.5 ng cm^{-2} yr^{-1} at present day. Lower

fluxes of ofloxacin were found in both Duluth Harbor (less than 0.7 ng cm^{-2} yr^{-1}) and Lake Pepin (less than 5 ng cm^{-2} yr^{-1}) after initial appearance in 1980 and 1990, respectively. Norfloxacin, a human-use drug, received FDA approval in 1986. It was detected once in Lake Winona around 2010, but was found in Duluth Harbor from 1980 to the present day. Ciprofloxacin, a fluoroquinolone generally consumed by humans, swine, and chickens, was approved by the FDA in 1987. Aside from an unexplained detection in 1935, accumulation rates in Lake Winona rose from 3.0 ng cm^{-2} yr^{-1} in 1980 to a present-day flux of 8.7 ng cm^{-2} yr^{-1}. Enrofloxacin, used by the agricultural industry, was not detected in any sample. Enrofloxacin is known to photo-transform into ciprofloxacin in surface waters and may contribute to ciprofloxacin accumulation.[199]

Macrolides. Of the three macrolides included in this study, only erythromycin was detected. In addition to mass production for human and animal use since 1972, erythromycin is also naturally produced. The presence of the erythromycin, however, has a level of uncertainty. The purity of isotopically labeled erythromycin, which was used as an internal standard, was 90%. Therefore, roughly 10 ng of unlabeled erythromycin was added to each sample. Erythromycin was typically detected in all samples, but most often the method blank would subtract off the contamination, e.g., no mass above the method blank was quantified in Little Wilson. For both Duluth Harbor and Lake Pepin, the appearance of erythromycin above the method blank was sporadic and likely due to the addition of the internal standard. Unlike the other cores, erythromycin was present in Lake Winona from 1950 to 2015 with fluxes ranging from 0.04 to 0.28 ng cm^{-2} yr^{-1}. Thus, it is likely that the detection of erythromycin Lake Winona derives from anthropogenic inputs rather than the addition of isotopically labeled internal standard.

The other macrolides included in this study, tylosin and roxithromycin, are not prescribed to humans and were not detected in any of the study lakes.

Tetracyclines. None of the tetracyclines included in this study were detected in the sediment cores. Even with the improved extraction efficiency with the UAE, no tetracyclines were detected in Lake Winona, the most heavily WWTP-impacted lake included in this study.

Non-categorized. Trimethoprim was first approved as a mixture with sulfamethoxazole in 1973 and was detected in all three wastewater-impacted lakes. The detection of trimethoprim in Lake Pepin and Duluth in 1990 was delayed several years relative to FDA approval, but trimethoprim appeared in Lake Winona around 1980, only a few years after receiving approval. Accumulations in Lake Pepin (0.7 to 1.2 ng cm^{-2} yr^{-1}) and Lake Winona (0.03 to 0.78 ng cm^{-2} yr^{-1}) were about 10-fold higher than Duluth Harbor (0.06 to 0.09 ng cm^{-2} yr^{-1}). Lincomycin is a naturally occurring antibiotic that is also mass produced for human and animal treatments. It was detected throughout the Lake Pepin sediment record with accumulation rates ranging from 0.04 to 1.8 ng cm^{-2} yr^{-1}. The occurrence of lincomycin did not appear to be affected by the 1964 FDA approval. Carbadox, primarily used by swine, was not detected in any of the lake sediment cores.

3.5 Discussion

Method Comparison. ASE was the initial extraction method used to extract and quantify antibiotic concentrations in the sediment. It produced low recoveries for select fluoroquinolones and tetracyclines. Lake Pepin and Lake Winona sediment cores were, therefore, re-extracted with the UAE method in an attempt to achieve higher recoveries.

Lake Pepin and Lake Winona sediment cores were chosen because they were the most heavily impacted by wastewater and therefore the most likely to accumulate antibiotics.

A new stationary phase, Waters XSelect CSH C18, was used for LC-MS/MS analysis of UAE extracts due to the broad peaks of tetracyclines with Phenomenex Kinetex F5 column. The peak broadening was thought to have been caused by interactions between the positive charge on tetracyclines and the negatively charged silanol on the particle core, thus a column with a positively charged surface was selected.

The UAE method was equal to or more efficient than ASE as an extraction method for all four tetracyclines and three out of the four fluoroquinolones. Tetracyclines and fluoroquinolones generally had similar or better detection limits using the UAE method when compared to ASE detection limits. UAE is also a viable option for extraction of sulfonamides, lincomycin, carbadox, and trimethoprim with sufficient, comparable or better recoveries than ASE and similar detection limits between the two extraction methods. Even with different extraction methods and stationary phases for LC-MS/MS analysis, the reported antibiotic accumulation rates were similar between the two methods in Lake Winona, (Figures 3.2 and C.9). Both extraction methods were reproducible given the similar accumulation rates between replicates.

Benefits of using the UAE method include not requiring an expensive instrument to maintain or the rigorous and time-consuming step of cleaning the ASE stainless-steel cells. Also, the chance of carry over contamination is reduced, because a new centrifuge tube is used to extract each sample. A major disadvantage of UAE is that it does not appear to be suitable for all types of sediment. A precipitate formed in the Lake Pepin UAE extract that resulted in non-detects of tetracyclines and fluoroquinolones, even in

the samples spiked with antibiotics. ASE appears to be a more robust and preferable extraction method, even with low recoveries for some tetracyclines and fluoroquinolones. The Waters XSelect CSH C18 column was successful in producing narrow tetracycline peaks (see Figure C.3) and in separating all the antibiotics included in this study. Also, this column did not require the flushing of a EDTA:methanol solution prior to analysis to maintain tetracycline and fluoroquinolone peak shapes which was necessary for the Phenomenex column. Thus, our research suggests that future analyses should use the ASE extraction coupled with a Waters XSelect CSH C18 column for the most robust extraction and analysis of antibiotics, although further testing is needed to confirm these observations.

Historical Trends. The sediment cores were successful in capturing historical trends in select antibiotic usage. In general, the appearances of antibiotics in the sediment cores were consistent with the initial FDA approval dates, which further validates results of the radiometric dating. In some profiles, the presence of an antibiotic was delayed from the approval date, such as sulfapyridine and trimethoprim. This suggests that either the popularity of the drug increased years after its FDA approval, there was a delay between FDA approval and administering to patients, the analytical method was not sensitive enough, or degradation occurred in the sediment.

On the other hand, some of the synthetic antibiotics in the Lake Winona core were present prior to their FDA approval. Their occurrence may be due to smearing from the topmost sediment of the core down during the collection process. The outer circumference of core was removed to reduce contamination during the collection process, but perhaps not enough was removed. It is also possible that some compounds

have limited downward mobility in the sediment bed.[200] This is more likely for compounds with lower sorption capacity, such as sulfonamides, relative to analytes that sorb more readily, e.g. tetracyclines and fluoroquinolones.[130,189,190] Furthermore, Tamtam et al [200] saw limited to non-existent mobility of sulfonamides and tetracyclines in their sediment record. Therefore, it is more likely that the collection process is the source of the contamination. This does not hinder interpretations regarding overall trends, as accumulation rates increased notably around drug approval dates. The presence of sulfamethazine in Duluth Harbor from 1920 to 1950 occurred within a core length of 8 cm. Its presence prior to FDA approval may be attributed to sediment mixing, perhaps related to nearby harbor dredging activities. It is unclear why this anomalous detection only occurred for this compound, however.

Quantifying historical levels of the ten detected antibiotics also indicates that these pharmaceuticals are relatively persistent in the sediment matrix. Even low levels of antibiotics in the environment are concerning, because studies have shown that sub-therapeutic levels may promote greater variety of antibiotic resistance over time.[147,148,201] The effect of antibiotics in sediments on the bacterial community is not fully understood and therefore needs further investigation.[4,149,202]

Usage Trends. Antibiotic profiles in sediment cores may provide insight into which drugs are most frequently prescribed. The World Health Organization's (WHO) list of essential medications indicate which pharmaceuticals are needed for a basic human health-care system. Six of the eight antibiotics that were on the WHO list and included in this study (sulfadiazine, sulfamethoxazole-trimethoprim, erythromycin, ciprofloxacin, and ofloxacin) were detected in at least one of the lakes. The WHO list may serve as a

catalog of frequently used drugs for which the fate and transport in the environment need to be more fully understood. The significance of tracking the fate and transport of heavily used drugs is demonstrated by the widespread presence of sulfapyridine after it fell into disuse. The accumulation of sulfapyridine is likely due to the consumption of sulfasalazine, a drug used to treat and prevent ulcerative colitis and treat rheumatoid arthritis and Crohn's disease.[112] Sulfasalazine is on the list of essential medications and was approved in 1950. Sulfapyridine is a metabolite of sulfasalazine. Thus, the accumulation of sulfapyridine in these lakes since 1950 is likely due to both direct use and the metabolism of sulfasalazine.

Comparing historical antibiotic accumulation rates may indicate which antibiotics are most prevalent and/or persistent. In the studied lakes, fluoroquinolone fluxes were greater than any other antibiotic class. According to a recent US study, fluoroquinolones were the most commonly prescribed in US hospitals.[203] It is also well known that fluoroquinolones sorb readily to sediment.[189,190] The high observed fluxes are likely due to both sorption and usage trends.

It was somewhat surprising to not detect tetracyclines in the sediment cores given their high affinity for solids. Because some tetracyclines are naturally produced, unlike sulfonamides and fluoroquinolones, it is possible that tetracyclines are more susceptible to transformations and therefore are detected less frequently. All tetracyclines have been shown to biodegrade,[204–206] and oxytetracycline can undergo hydroloysis.[4] Tetracyclines are also photochemically labile in surface waters,[207,208] as well as while sorbed to minerals.[209]

In the popular drug combination of trimethoprim and sulfamethoxazole, trimethoprim accumulated at higher concentrations and rates than sulfamethoxazole. These two synthetic, human-use drugs have been typically prescribed in a 1:5 trimethoprim:sulfamethoxazole ratio since 1973. The lack of sulfamethoxazole may be due to other fate processes that are less significant for trimethoprim, such as sulfamethoxazole being more photolabile.[210] This was also demonstrated by a recent study that detected both sulfamethoxazole and trimethoprim in surface water, but only trimethoprim was found in sediment beds.[128] Zhou et al.[124] also did not detect sulfamethoxazole in the presence of trimethoprim in river sediments. Other studies have reported higher levels and frequency of trimethoprim in sediment compared to sulfamethoxazole.[131,211] The sedimentary record suggests the trimethoprim is more persistent in sediment and therefore may be of greater concern.

Natural vs Synthetic. Of the antibiotics known to be natural products (erythromycin, tylosin, lincomycin, chlortetracycline, tetracycline, and oxytetracycline), two were detected in the sediment cores. Erythromycin was present in Lake Winona prior to its FDA approval; but as previously mentioned, a limitation of this study was the addition of erythromycin while spiking in the isotopically labeled erythromycin. In the other cores, none or sporadic samples had levels of erythromycin above the method blank. Lake Winona was the only core that had concentrations above the method blank throughout most of the core. It is possible that erythromycin was naturally produced in Lake Winona, but unlikely. On the other hand, the dominant source of the lincomycin accumulating in Lake Pepin may be natural production. This antibiotic was detected pre-FDA approval and accumulation did not noticeably change after approval. It is unclear

why the peak at around 1940 is present, but it is possible water conditions at the time were conducive to its natural production. Lincomycin is primarily used by the agriculture industry (e.g. poultry and swine), unless a patient has an infection resistant to penicillin. The limited detection of naturally produced antibiotics overall suggests that, as would be expected, they are more susceptible to degradation than synthetic antibiotics.

Human vs Agricultural Activity. Wastewater effluent appeared to be the primary source of antibiotic pollution in the anthropogenically-impacted lakes. Antibiotics that were partially or completely used for human treatments were predominately detected. It is likely that a portion of the antibiotics accumulating in Lake Winona and Lake Pepin are derived from animal use, given the extent of agricultural activity in their watersheds. The frequent detection of antibiotics on the WHO list of essential medications also indicates wastewater effluent as the primary source.

The degree of antibiotic pollution also appears to reflect the degree of wastewater impact. In general, the highest fluxes and greatest number of antibiotics were measured in Lake Winona, followed by Lake Pepin, and Duluth Harbor in decreasing order. Antibiotics were not detected at the control site, Little Wilson Lake. The Minnesota Pollution Control Agency reported that from 2000 to 2008, approximately 63% of the average inflow to Lake Winona was wastewater effluent from Alexandria Lakes Area Sanitation District WWTP (3.75 MGD).[212] It was not surprising, therefore, that antibiotic pollution was greatest in Lake Winona because it was also the most heavily impacted by treated wastewater.

The Duluth Harbor core was expected to record more antibiotic pollution than in Lake Pepin, because the highest levels of human-use antibiotics are generally seen in

surface waters and sediments near wastewater outfalls.[123,127,128] The Duluth Harbor core was collected 5 km from the outfall of Western Lake Superior Sanitary District WWTP (40 MGD) and less than 1 km from Superior, Wisconsin WWTP (5 MGD). Lake Pepin is a natural impoundment of the Mississippi River on the Minnesota-Wisconsin border and downstream of the convergence of the Minnesota and Mississippi River. In addition to receiving upstream inputs from the Metropolitan WWTP (170 MGD) and other smaller municipalities, Lake Pepin receives direct wastewater discharges from Red Wing, WI (3 MGD) and Lake City, MN (1.8 MGD) 22 km upstream and 6 km downstream, respectively, of the collection site. As shown in Figure 3.1, higher fluxes were observed in Lake Pepin than in Duluth Harbor, a likely consequence of Lake Pepin's large watershed (half of Minnesota) and extremely high sediment load, which may carry sorbed antibiotics. Thus, the accumulation rates of antibiotic in Lake Pepin may incorporate antibiotic usage along the Minnesota and upper Mississippi Rivers.

The ability of antibiotics to migrate downstream likely explains the presence of sulfamethazine in Lake Pepin. The presence of the animal-use medicine in Lake Pepin may be due to upstream agricultural activity along the Minnesota and Mississippi rivers. The detection of sulfamethazine in Lake Winona indicates that agricultural usage in the surrounding watershed was captured by the sediment record as well. Approximately 30% of its watershed is cultivated crop land where manure may be applied.

It is also interesting to note that carbadox, which is only administered to swine, was not detected in any of the sediment samples. This is in contrast to a recent study that found carbadox in 28% of the 50 Minnesota lakes and streams sampled.[2] The extraction/analysis method (LOD range from 0.09 to 1.24 ng/g of sediment) and

experimental sorption capacity to organic matter (log Koc 3.96 ± 0.18 L/kg OC) appears sufficient for accumulation and detection of the antibiotic.[213] The absence of carbadox could be due to fate processes in the water column or benthic sediments that degraded the antibiotic. Another possibility is that the detection of carbadox in the previous study is a false positive, because the ASE method used in this work saw a large peak at a similar retention time to carbadox in the quantification SRM that was not present in the confirmation SRM and was therefore not quantified. The unknown peak in the quantification SRM designated for carbadox was sufficiently large enough a peak in the confirmation SRM should have been present.

Environmental Implications. This research suggests that wastewater impacted lakes capture the trends of antibiotic usage predominantly in human medicine. Human-use antibiotics present in lakes originate largely from WWTP effluent and therefore have a direct point-source route into these systems. Agricultural/veterinary antibiotic trends are not as readily captured in these lake systems, even when their watersheds contain animal feeding operations where antibiotics are used or cropland where antibiotic-contaminated manure may be spread. Only in Lake Winona, which has a relatively small watershed, were antibiotics used in agriculture routinely detected. While a recent study estimated that a majority of the antibiotics consumed in the US are for agricultural activities,[3] land application of agricultural antibiotics may limit their transport and accumulation into the studied lakes. Although, lakes appear to be suitable locations to evaluate historical loading trends of antibiotics used in human medicine via their direct input from WWTPs, our study suggests that accumulation rates and impacts due to antibiotic use in agriculture requires sampling in soils or waterways near agricultural activities (rather than in lakes

that integrate signals from large watersheds) due to the non-point source route of agricultural antibiotics to the environment.

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