# Final Abstract

Final Report Approved on November 21, 2024

## M.L. 2021 Project Abstract

For the Period Ending June 30, 2024

**Project Title:** Antibiotic Resistance and Wastewater Treatment: Problems and Solutions

**Project Manager:** Justin Donato

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**Funding Source:**

**Fiscal Year:**

**Legal Citation:** M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 04j

**Appropriation Amount:** $432,000

**Amount Spent:** $410,177

**Amount Remaining:** $21,823

**Sound bite of Project Outcomes and Results**

We tracked and quantified antibiotic resistance genes (ARGs) through the municipal wastewater treatment process at full-scale treatment facilities. This analysis was conducted at multiple time points, generating a comprehensive picture of the dynamic changes in abundance and diversity of ARGs for assessment of the potential for their environmental spread.

**Overall Project Outcome and Results**

Antibiotic resistant bacteria (ARB) represent an increasingly prevalent health threat. Taking a One Health approach, it is necessary to understand the factors that facilitate the spread and the destruction of ARB in human, animal, and environmental settings as they all impact each other. In this study, we focused on the bacteria associated with the municipal wastewater treatment process. ARB are among the many contaminants in wastewater as it arrives at facilities for treatment. We studied the bacteria within wastewater and the resulting biosolids, as it moved through the treatment process, focusing on the antibiotic resistance genes (ARGs) they harbor. Our main question was whether the ARB were eliminated during the treatment process, or if they thrived and spread ARGs to neighboring bacteria, thus creating superbugs. We analyzed ARGs from millions of DNA sequences, derived from thousands of bacteria. We found

ARGs generally decreased in abundance and diversity as the wastewater traversed the treatment process (moving from raw influent to aerobic bioreactor to anaerobic digester), regardless of the treatment technology employed. This shift in abundance was clearly seen as many bacteria related to potential pathogens in the influent were reduced to undetectable levels during the treatment process. As expected, incineration reduced ARGs to undetectable levels in the resulting ash samples. Fortunately, our analyses did not detect any evidence of dissemination of ARGs among bacteria that led to the creation of superbugs. However, we detected spread of other DNA sequences that were unrelated to antibiotic resistance. Therefore, although we didn't see ARGs spread, the threat for their future dissemination is possible. Future research will focus on characterizing the DNA molecules that are spreading among bacteria to more fully understand the factors leading to their dissemination, facilitating the assessment of the threats of spread of ARGs in Minnesota's municipal wastewater systems.

**Project Results Use and Dissemination**

The dissemination efforts include three main activities. First, the findings have been presented at multiple scientific conferences for experts in the field to engage research. Second, the study has been submitted for publication in a peer-reviewed journal. The manuscript is in the final stages of the review process. Once published, the paper will be offered as open-access, so readers will not need to pay for access to the journal. Finally, the data generated in this study has been made publicly available in the National Center for Biotechnology Information's database that is freely accessible to anyone looking to analyze the data.

# Environment and Natural Resources Trust Fund

M.L. 2021 Approved Final Report

## General Information

**Date:** November 22, 2024

**ID Number:** 2021-390

**Staff Lead:** Mike Campana

**Project Title:** Antibiotic Resistance and Wastewater Treatment: Problems and Solutions

**Project Budget:** $432,000

## Project Manager Information

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## Project Reporting

**Final Report Approved:** November 21, 2024

**Reporting Status:** Project Completed

**Date of Last Action:** November 21, 2024

**Project Completion:** June 30, 2024

## Legal Information

**Legal Citation:** M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 04j

**Appropriation Language:** $432,000 the first year is from the trust fund to the commissioner of natural resources for an agreement with the University of St. Thomas to quantify the ability of full-scale wastewater treatment plants to eliminate antibiotic resistance genes entering or created in the water treatment process before these genes are released into the natural environment.

**Appropriation End Date:** June 30, 2024

## Narrative

**Project Summary:** This project will quantify the ability of full-scale wastewater treatment plants to eliminate antibiotic resistance genes and the extent to which these genes are exchanged during the wastewater treatment process.

**Describe the opportunity or problem your proposal seeks to address. Include any relevant background information.**

Pandemic infectious diseases are an increasing threat to our daily lives. At present, more than 35,000 people die of antibiotic-resistant infections each year in the United States; some models are predicting as many as 10 million annual deaths attributable to antibiotic resistant infections (worldwide) by 2050. The scientific community now understands that untreated municipal wastewater (sewage) is especially rich in antibiotic resistant bacteria.

We postulate, therefore, that wastewater treatment is a potential solution to the problem of antibiotic resistance, although we expect different systems to perform better than others. This project, therefore, proposes to investigate full-scale wastewater treatment facilities to determine which designs are best-suited to eliminate antibiotic resistant bacteria.

Almost paradoxically, we also postulate that an unexpected consequence of centralized municipal wastewater treatment is that it facilitates the development of novel bacteria that are simultaneously resistant to multiple antibiotics. Bacteria are well-known to be able to exchange genetic material, particularly when there are dense communities of different microorganisms – which are precisely the conditions that are intentionally created during and are essential to the success of biological wastewater treatment.

**What is your proposed solution to the problem or opportunity discussed above? Introduce us to the work you are seeking funding to do. You will be asked to expand on this proposed solution in Activities & Milestones.**

We hypothesize that sewage sludge incineration, which is performed at the Metropolitan Wastewater Treatment Plant in St. Paul, is the best and most cost-effective technology for eliminating antibiotic resistant bacteria in untreated municipal wastewater. The application of incineration to treat sewage sludge is relatively rare in the United States but common in other countries. The reason that sewage sludge incineration is rarely practiced in the United States is due to obsolete concerns regarding air pollution; modern incinerators pose little threat to air pollution, such that a country like Switzerland (with some of the most stringent environmental regulations in the world) incinerates the majority of its sewage sludge.

Although it is inarguable that municipal wastewater treatment is necessary to protect surface water quality and public health, it is not without unanticipated consequences. The creation of a nearly ideal environment for bacteria to exchange genetic material is likely one of these collateral consequences. Common wastewater bacteria are known to exchange antibiotic resistance genes when grown as isolated cultures. However, this ability has yet to be fully explored in mixed bacterial communities present at wastewater treatment facilities. We will track resistance genes within these communities to monitor and eventually eliminate their dissemination.

**What are the specific project outcomes as they relate to the public purpose of protection, conservation, preservation, and enhancement of the state's natural resources?**

This project takes a ONE HEALTH perspective, which is defined as a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment. The implementation of the findings from this project will lead to more efficient removal of antibiotic resistance genes from untreated wastewater. If our hypothesis is correct, we anticipate a gradual shift towards the increased application of incineration for treated sewage sludge. This project, therefore, should lead to better environmental and public health.

## Project Location

**What is the best scale for describing where your work will take place?**

Statewide

**What is the best scale to describe the area impacted by your work?**

Statewide

**When will the work impact occur?**

During the Project and In the Future

## Activities and Milestones

### Activity 1: Quantify the ability of different wastewater treatment facilities to remove/eliminate antibiotic resistance genes.

**Activity Budget:** $307,000

**Activity Description:**
Samples will be collected from multiple locations within municipal wastewater treatment facilities to allow us to determine the fraction of antibiotic resistant bacteria that enter the facility (i.e., with raw sewage), exit the facility with the treated effluent (i.e., the "clean" wastewater that is usually released to a river), and the treated sewage solids (i.e., either incinerated, applied to agricultural land, or landfilled). DNA will be extracted and purified from these samples and we will use techniques pioneered at the University of Minnesota to quantify numerous genes known to encode antibiotic resistance. We would like to work with at least 10 different wastewater treatment plants and to collect at least triplicate samples from each facility (i.e., samples collected on different dates). We anticipate collect from 6 different locations within each treatment facilities (i.e., we anticipate collecting at least 180 samples; 10 facilities x 3 sample dates x 6 locations = 180). We anticipate quantifying at least 20 different antibiotic resistance genes.

**Activity Milestones:**

| Description | Approximate Completion Date |
|---|---|
| Sample collection and processing | December 31, 2022 |
| Quantification of antibiotic resistance genes | August 31, 2023 |
| Data Analysis | December 31, 2023 |

### Activity 2: Recognizable antibiotic resistance genes from each stage of wastewater treatment will be identified and tracked as they spread between bacteria.

**Activity Budget:** $81,215

**Activity Description:**
Antibiotic resistance genes will be identified by analyzing all DNA sequences present in wastewater-derived samples. A subset of the samples harvested in Activity 1 will be subjected to a more comprehensive, cutting-edge DNA sequencing technology that is commercially available from Phase Genomics. Antibiotic resistance genes are often located on small DNA segments that are exchanged between bacteria. Therefore, identifying which bacterial species harbors a given resistance gene by simply analyzing the isolated DNA fragments is often impossible. The technology that will be used here links resistance gene sequences to their bacterial hosts, facilitating identification of (1) all resistance genes in the samples and (2) which bacteria harbor those resistance genes. Samples will be taken from four points within four treatment facilities. All of the DNA within each sample will be fully sequenced, yielding billions of base pairs of genetic data. The resistance genes within this dataset will be linked to the specific identities of bacteria in each original sample. Since samples will be taken from multiple points in the treatment process, changes in the identities of the bacteria associated with specific resistance genes will indicate spread of those resistance genes during wastewater processing.

**Activity Milestones:**

| Description | Approximate Completion Date |
|---|---|
| DNA processing and submission for sequencing | June 30, 2022 |
| Antibiotic resistance gene determination | June 30, 2023 |
| Spread of resistance gene analysis complete | June 30, 2024 |

## Activity 3: Use lab-scale reactors to directly compare wastewater treatment technologies with a common influent.

**Activity Budget:** $43,785

**Activity Description:**

The findings from Activity 2 will include differences that are due to differences between the technologies (the goal of this project) and variability between the sources of raw sewage. When comparing two full-scale facilities, this second type of variability is unavoidable. However, in lab-scale experiments, a single influent source can be used to feed experiments that test differences between the two technologies. We will set up six lab-scale reactors (three for each technology) that are fed by primary/secondary solids from the first full-scale facility described in Activity 2. Each of the reactors will be sampled at three points for a total of 18 reactor samples. Three samples of the influent will also be included in the analysis. All 21 samples will be sent to Phase Genomics for Hi-C DNA sequencing and analysis. The resulting data will include triplicate analyses of the microbiome, and specifically, the antibiotic resistance genes and mobile genetic elements present in the samples. These data will substantially strengthen the conclusions reached from Activity 2.

**Activity Milestones:**

| Description | Approximate Completion Date |
|---|---|
| DNA sequence analysis | June 30, 2024 |

## Project Partners and Collaborators

| Name | Organization | Role | Receiving Funds |
|---|---|---|---|
| George Sprouse and Larry Rogacki | Metropolitan Council, Environmental Services | MCES will provide access to samples from the largest municipal wastewater facilities in the State of Minnesota. | No |
| Timothy LaPara | University of Minnesota - Twin Cities | Co-project manager; Dr. LaPara will co-supervisor the students and post-doc working on this project. | Yes |

## Dissemination

**Describe your plans for dissemination, presentation, documentation, or sharing of data, results, samples, physical collections, and other products and how they will follow ENRTF Acknowledgement Requirements and Guidelines.**
Findings will be shared among the project personnel. This includes the Metropolitan Council Environmental Services, so the information will be available to wastewater facility managers they oversee. The findings will be included in reports to LCCMR, peer-reviewed publications, conference presentations, and potentially press releases to the media. In presentations with a visual component (e.g. conference presentations with slides and poster presentations), the ENRTF logo will be displayed. In those instances, ENRTF will also be acknowledged orally. In print media (e.g. peer-reviewed publications), a statement acknowledging ENRTF will be included per the ENRTF guidelines. The DNA sequence data generated from this work will be deposited in GenBank, a publicly available repository. Where appropriate, the associated metadata and sequence data will also be made publicly available in databases such as CARDLive for use by other researchers. The intended audience includes the public, wastewater treatment managers, and scientists.

## Long-Term Implementation and Funding

**Describe how the results will be implemented and how any ongoing effort will be funded. If not already addressed as part of the project, how will findings, results, and products developed be implemented after project completion? If additional work is needed, how will this work be funded?**
The findings from this project will inform the best practices for eliminating antibiotic resistance genes during the wastewater treatment process. The processes being analyzed in this project are already in place at different municipal wastewater treatment facilities. Once the most efficient processes have been established, the information will be shared with facility managers to use in determining their preferred methods of antibiotic resistance gene elimination. We anticipate new lines of research may be stimulated by the proposed project; we will seek funding for these ideas from appropriate sources, such as the National Science Foundation and the LCCMR/MN ENTRF.

## Other ENRTF Appropriations Awarded in the Last Six Years

| Name | Appropriation | Amount Awarded |
|---|---|---|
| Triclosan Impacts on Wastewater Treatment | M.L. 2014, Chp. 226, Sec. 2, Subd. 03c | $380,000 |

## Budget Summary

| Category / Name | Subcategory or Type | Description | Purpose | Gen. Ineligible | % Benefits | # FTE | Classified Staff? | $ Amount | $ Amount Spent | $ Amount Remaining |
|---|---|---|---|---|---|---|---|---|---|---|
| **Personnel** | | | | | | | | | | |
| Undergraduate Researcher | | Researcher | | | 8% | 0.99 | | $31,000 | - | - |
| Postdoctoral Researcher | | Researcher | | | 33% | 3 | | $201,215 | - | - |
| Justin Donato | | Project Manager | | | 8% | 0.24 | | $27,400 | - | - |
| | | | | | | | **Sub Total** | **$259,615** | **$250,950** | **$8,665** |
| **Contracts and Services** | | | | | | | | | | |
| Phase Genomics | Professional or Technical Service Contract | This project is dependent upon the generation of DNA sequence datasets using a technology pioneered by Phase Genomics. We plan to generate these data as a fee for service. Parts of the data that will be generated by the University of Minnesota Genomics Center to reduce costs if possible. | | | | 0 | | $93,785 | $92,985 | $800 |
| University of Minnesota - Twin Cities | Sub award | This sub-award will fund the activities conducted by Dr. Timothy LaPara, the co-PI on this project. These funds will cover expenses associated with compensation for Dr. LaPara and one researcher, lab supplies, and travel within MN for sample collection. | | | | 0.15 | | $59,600 | $55,616 | $3,984 |
| | | | | | | | **Sub Total** | **$153,385** | **$148,601** | **$4,784** |
| **Equipment, Tools, and Supplies** | | | | | | | | | | |
| | Tools and Supplies | Reagents for molecular biology analysis of antibiotic resistance genes | Antibiotic resistance genes will be detected and quantified using standard techniques and reagents. | | | | | $6,000 | $3,688 | $2,312 |
| | | | | | | | **Sub Total** | **$6,000** | **$3,688** | **$2,312** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Capital Expenditures** | | | | | | | | | | |
| | | One autoclave | The majority of tools and reagents to complete this project need to be sterilized in an autoclave. | | | | | $8,000 | $6,938 | $1,062 |
| | | | | | | | **Sub Total** | **$8,000** | **$6,938** | **$1,062** |
| **Acquisitions and Stewardship** | | | | | | | | | | |
| | | | | | | | **Sub Total** | **-** | **-** | **-** |
| **Travel In Minnesota** | | | | | | | | | | |
| | | | | | | | **Sub Total** | **-** | **-** | **-** |
| **Travel Outside Minnesota** | | | | | | | | | | |
| | | | | | | | **Sub Total** | **-** | **-** | **-** |
| **Printing and Publication** | | | | | | | | | | |
| | Publication | Open access journal article publishing | Dissemination of findings | | | | | $5,000 | - | $5,000 |
| | | | | | | | **Sub Total** | **$5,000** | **-** | **$5,000** |
| **Other Expenses** | | | | | | | | | | |
| | | | | | | | **Sub Total** | **-** | **-** | **-** |
| | | | | | | | **Grand Total** | **$432,000** | **$410,177** | **$21,823** |

## Classified Staff or Generally Ineligible Expenses

| Category/Name | Subcategory or Type | Description | Justification Ineligible Expense or Classified Staff Request |
|---|---|---|---|

## Non ENRTF Funds

| Category | Specific Source | Use | Status | $ Amount | $ Amount Spent | $ Amount Remaining |
|---|---|---|---|---|---|---|
| **State** | | | | | | |
| | | | **State Sub Total** | **-** | **-** | **-** |
| **Non-State** | | | | | | |
| In-Kind | University of Minnesota - Twin Cities | These are overhead contributions that would otherwise be requested as indirect costs. | Secured | $32,200 | $32,200 | - |
| In-Kind | University of St. Thomas | These are overhead contributions that would otherwise be requested as indirect costs. | Secured | $164,400 | $164,400 | - |
| | | | **Non State Sub Total** | **$196,600** | **$196,600** | **-** |
| | | | **Funds Total** | **$196,600** | **$196,600** | **-** |

## Attachments

### Required Attachments

*Visual Component*

File: aea5dc97-e00.pdf

*Alternate Text for Visual Component*

The problem of antibiotic resistance gene spread among wastewater bacteria can be eliminated through effective treatment strategies. Technologies including incineration can be implemented to destroy antibiotic resistant bacteria....

*Financial Capacity*

File: 81d06e83-756.pdf

### Supplemental Attachments

*Capital Project Questionnaire, Budget Supplements, Support Letter, Photos, Media, Other*

| Title | File |
|---|---|
| Research Addendum | 34e24645-d73.docx |
| Signed Background Check | 0b967eda-b4b.pdf |
| MN One Health Antibiotic Stewardship Consortium Presentation | dac62143-417.pdf |
| American Society for Microbiology Presentation | 49e31a89-e10.pdf |
| Poster Presentation | 161cb0e8-013.pdf |

## Difference between Proposal and Work Plan

*Describe changes from Proposal to Work Plan Stage*

To meet the reduced budget, one undergraduate was removed from the project, the project manager compensation was reduced, and the subaward amount was reduced.

## Additional Acknowledgements and Conditions:

The following are acknowledgements and conditions beyond those already included in the above workplan:

**Do you understand and acknowledge the ENRTF repayment requirements if the use of capital equipment changes?**

Yes

**Do you agree travel expenses must follow the "Commissioner's Plan" promulgated by the Commissioner of Management of Budget or, for University of Minnesota projects, the University of Minnesota plan?**

N/A

**Does your project have potential for royalties, copyrights, patents, sale of products and assets, or revenue generation?**

No

**Do you understand and acknowledge IP and revenue-return and sharing requirements in 116P.10?**

N/A

**Do you wish to request reinvestment of any revenues into your project instead of returning revenue to the ENRTF?**

N/A

**Does your project include original, hypothesis-driven research?**

Yes

**Does the organization have a fiscal agent for this project?**

No

# Work Plan Amendments

| Amendment ID | Request Type | Changes made on the following pages | Explanation & justification for Amendment Request (word limit 75) | Date Submitted | Approved | Date of LCCMR Action |
|---|---|---|---|---|---|---|
| 1 | Amendment Request | • General Information<br>• Budget - Personnel<br>• Activities and Milestones<br>• Budget - Professional / Technical Contracts | We have an opportunity to substantially increase the rigor of our scientific conclusions by performing analogous DNA sequencing by Phase Genomics on samples collected from a replicated, lab-scale anaerobic digester experiment in which the same source material (sewage sludge) was used. This additional analysis would cost $43,785; our budget can afford these additional costs because we have underspent on the salary category (undergraduate and postdoctoral researcher) by a projected $50,000. | February 26, 2024 | Yes | February 28, 2024 |

# Status Update Reporting

## Final Status Update August 14, 2024

**Date Submitted:** October 30, 2024

**Date Approved:** October 31, 2024

**Overall Update**

We have now completed the data generation for all activities of this project. We have also completed the analyses of those data. We have tracked antibiotic resistance genes (ARG) abundance and diversity throughout the wastewater treatment process at full scale treatment facilities. We have also catalogued the bacteria present at multiple points in the treatment process across the treatment facilities. All this work was done at multiple time points to yield a comprehensive view of the patterns of antibiotic resistance present, the identities of the bacteria harboring those resistance genes, and the degree with which those ARG threats are removed during the treatment process. We used different technologies to address these questions to gain a broad understanding of the dynamics of the antibiotic resistome across all samples. We have detected the transfer of DNA between bacteria; however, we have yet to detect ARG transfer. In total, the general trend that we observed is the decrease in both the abundance and diversity of the ARGs as material traverses the wastewater treatment process. As a result of this project, our findings have been presented at multiple scientific conferences. We have also submitted one manuscript for publication in a peer-reviewed scientific journal.

**Activity 1**

We used quantitative PCR, to assess the abundances of bacteria, specific ARG, and genes known to be associated with the spread of genetic information between bacteria. This technology was employed as it enabled us to analyze trends across hundreds of samples; an accomplishment that would have been cost prohibitive using the more advanced DNA sequencing technology associated with Activity 2. The data from this activity provided a broad picture of the efficiency with which ARG are removed from wastewater. Across the samples, we observed that ARG as well as the gene associated with the spread of ARG decrease at material traverses the wastewater treatment process so that the highest numbers were observed in the untreated wastewater and the lowest abundances were observed in the effluent. These findings were consistent across the wastewater technologies included in this study. While incineration resulted in the most extensive removal of ARG, there are other factors (e.g. air pollution) that may impact the implementation of this technology in full-scale treatment facilities. The results were presented orally at a scientific conference, and a scientific publication is currently being prepared with the intention of submitting the results to a peer-reviewed journal.
*(This activity marked as complete as of this status update)*

**Activity 2**

The work in this activity complements the work in Activity 1. Whereas the achievements of Activity 1 yielded a pattern of abundances of bacteria and specific genes across many samples, the efforts associated with Activity 2 focused on fewer samples, but in much greater depth. In this Activity, we used cutting-edge DNA sequencing technology to gain information on more than just the abundance of a select few genes. Instead, we generated data on all genes present, and we mapped those genes onto specific genomes to facilitate the identification of specific bacteria. We now have a comprehensive census of the antibiotic resistance genes present in all samples, as well as the identifiable bacteria in each sample as well; even those present at levels that were too low to detect using previously available technologies. Our analyses have shown the wastewater treatment process to be effective at removing specific bacteria of concern to human health to levels that were below our detection. Many of those bacteria harbor ARG, and we observed the abundance and diversity of those ARG decreasing as material traverses the treatment process as well. A manuscript detailing this work is undergoing peer review as part of the normal publication process.
*(This activity marked as complete as of this status update)*

**Activity 3**

The work in this activity complemented Activity 2, some of which enabled a comparison between two technologies (mesophilic anaerobic digestion; thermophilic anaerobic digestion) used to treat municipal wastewater solids. A limitation of Activity 2, however, was that two different wastewater treatment facilities were analyzed, meaning that subtle differences between the facilities (e.g., the composition of the untreated wastewater) could have led to significant differences in the results. Activity 3, therefore, was performed using a single source of wastewater solids, which was then treated using bench-scale bioreactors operated at 95 degrees F (mesophilic anaerobic digestion) and at 130 degrees F (thermophilic anaerobic digestion). Bioreactor samples were analyzed by the same cutting-edge DNA sequencing technology as was used in Activity 2. Our results largely confirmed the results of Activity 2 (that ARG abundance decreases as a result of treatment). In the lab scale reactors, the thermophilic digesters removed ARG more extensively than mesophillic digesters. Additional research is needed to understand the significance of this result. The bacterial communities were significantly different in the mesophilic anaerobic digesters compared to the thermophilic anaerobic digesters. Both types of anaerobic digesters resulted in a significant decrease in antibiotic resistance genes compared to the untreated wastewater.

*(This activity marked as complete as of this status update)*

**Dissemination**

The findings from this project have been publicized in multiple venues throughout the duration of the work. The research team have promoted this work through oral and poster presentations at a total of seven different scientific conferences. These include invited oral presentations at the MN One Health Antimicrobial Stewardship Consortium's annual meeting and the American Society for Microbiology's Microbe 2023 annual meeting. The work was also presented in poster form at the "Microbes in Wastewater: Molecular Approaches in Pathogen and AMR Surveillance" symposium as well as other local venues. We have prepared and submitted a manuscript for publication of this work. The manuscript is currently undergoing peer review as part of the publication process. The manuscript was submitted as an open access publication to facilitate its dissemination to a broader audience by removing the necessity that readers pay for access to the paper. The DNA sequence data have been deposited in the National Center for Biotechnology Information's comprehensive database. Those data are now freely available to anyone who wants to download and analyze the data for themselves.

# Status Update Reporting

## Status Update January 1, 2024

**Date Submitted:** January 22, 2024

**Date Approved:** February 6, 2024

**Overall Update**

Our analyses have reached the point where we can see patterns emerging that detail the fates of antibiotic resistance genes and the bacteria that harbor them as they move through the municipal wastewater treatment process. We have completed our census the antibiotic resistance genes present in all samples, and we have also catalogued the identifiable bacteria in each sample as well. These data sets include unprecedented levels of detail that capture information about the bacteria and their genes even at very low abundance. Overall, regardless of the wastewater treatment technology being employed, we generally see the abundance and diversity of the antibiotic resistance genes decreasing because of the treatment process. We are currently focusing on looking more closely, beyond the general trends, to focus on the behavior of specific bacteria and specific antibiotic resistance genes to gain a deeper understanding of how each behaves throughout the process.

**Activity 1**

Since the filing of our last progress report, we have now completed the DNA extraction and measurement of gene abundances for all samples associated with this study. We have completed this process for multiple antibiotic resistance genes as well as for genes associated with the transmission of antibiotic resistance genes and those used for calculating bacterial abundances. The entire dataset is now being analyzed. We have raw data on the abundances of all genes listed above, and we are comparing them to identify any that are anomalously high or low. The data collection and analyses for those samples will be replicated to ensure their accuracy. We anticipate the need to re-run a very small number of samples at this point. As noted in our previous report, we have found differences in gene abundances between samples. We are currently working to understand the impact of those differences on the efficiency of our wastewater treatment facilities to remove antibiotic resistance genes. The abundances we have uncovered also support the findings from activity 2 using the whole genome sequencing datasets.

**Activity 2**

At this point, we have completed our census of the diversity and abundance of antibiotic resistance genes in all samples. As often as possible, the association of those genes with specific bacteria has been identified. Now that we know which bacteria harbor each antibiotic resistance gene, we are focusing our attention on identifying the patterns that potentially indicate the spread of antibiotic resistance genes. We are cataloguing those genes that are associated with different bacteria at different times throughout the wastewater treatment process. We are also working to identify which genes are associated with genetic signatures that are known to be hallmarks for the exchange of genes between bacteria. We have found those genetic markers, and we are now working on combining that information with the information about the host bacteria throughout time to identify those antibiotic resistance genes that show evidence of having spread from one bacterium to another.

**Dissemination**

We continue to share the findings from this study. In addition to the conference presentations noted in our last report, we plan to present our findings at two upcoming meetings. The PI on this project will speak at the MN One Health Antimicrobial Stewardship Consortium's annual meeting, and the postdoctoral researcher on this project will present at the "Microbes in Wastewater: Molecular Approaches in Pathogen and AMR Surveillance" symposium. We also have two manuscripts that are nearing completion, and that we anticipate will be submitted for publication soon.

# Status Update Reporting

## Status Update July 1, 2023

**Date Submitted:** July 7, 2023

**Date Approved:** July 11, 2023

**Overall Update**

We continue to move closer to fully achieving the goals that we set when we proposed this project. Specifically, we have learned a great deal about the antibiotic resistance patterns present in the wastewater treatment process. We have identified many of the bacteria harboring resistance genes, the resistance genes themselves, and we have quantified the abundances of both the bacteria and the resistance genes. We have uncovered previously unknown diversity in the bacteria present as well as in the antibiotic resistance genes they harbor. The next stage in our analyses will be to use the diversity and abundance data to gain a better understanding of how the prevalence of antibiotic resistant bacteria and their genes changes throughout the wastewater treatment process. This work has entailed a minor change to the plan for allocating the funds in the subaward to the University of Minnesota. Dr. LaPara has devoted more time to this project than we originally anticipated, so salary that was originally budgeted for undergraduates has been reallocated to Dr. LaPara. This change did not affect the overall subaward amount, and it was approved by LCCMR staff.

**Activity 1**

At our last progress report, we had collected all samples for this activity, and the DNA needed for the analyses had been extracted from all samples. Since that time, we have continued our efforts to quantify the bacteria, the antibiotic resistance genes, and the genes associated with the spread of antibiotic resistance genes. We have one more tetracycline resistance gene that we plan to quantify. Once those data have been collected, we will analyze all the abundance data to determine if we need to look more closely at any additional tetracycline resistance genes, or if we should shift our focus to the characterizing the abundances of genes that confer resistance to different classes of antibiotics. We remain on pace to complete this data collection in accordance with the proposed milestones. The data collected to date indicate differences between sampling sites and locations. Our future analyses will help us to understand the significance of these differences. The initial analyses of the data associated with activity 2 of this project have also offered confirmatory data to support the initial findings in this analysis.

**Activity 2**

This activity will enable us to use DNA sequencing technology offered by Phase Genomics to track specific bacteria harboring specific antibiotic resistance genes throughout the wastewater treatment process. We have been applying this technology to study two wastewater treatment facilities that use different technologies. All the samples have been processed by Phase Genomics, and the resulting data have been made available to the research team. We have focused our initial efforts on cataloging the identities of the bacteria present in each sample as well as the antibiotic resistance genes present. We have found interesting patterns of variation in the diversity of bacteria and resistance genes as related to location in the treatment facility and over time. We are now working to understand the functional consequences of those differences in addition to our ongoing comparative analyses of the findings between the two wastewater facilities. We have also begun our initial analysis of the presence of the genes associated with the spread of resistance genes between bacteria. Together, we will use all these findings to gain a more complete understanding of the changes in the community structure and antibiotic resistance patterns throughout these treatment processes.

**Dissemination**

Our initial analyses have yielded enough significant findings for us to begin disseminating the results. The postdoctoral researcher associated with this project has presented her work in two venues. The first was at the Minnesota Supercomputing Institute's 2023 Research Computing Symposium, and the second was at the Microbe 2023 conference

of the American Society for Microbiology. The PI associated with this project also presented the work in a separate session at the Microbe 2023 conference of the American Society for Microbiology.

# Status Update Reporting

## Status Update January 1, 2023

**Date Submitted:** January 10, 2023

**Date Approved:** January 20, 2023

**Overall Update**

We have made significant progress towards the completion of the goals of this project. Our aims are to quantify antibiotic resistance genes and to characterize the spread of each resistance gene at multiple points in the wastewater treatment process from various treatment facilities around MN. Over the course of the last year, we have completed the necessary sample collection from wastewater treatment facilities distributed throughout MN. The processing steps that are required to extract the DNA from these samples has been completed for all samples in this study. We are currently analyzing the bacteria present in each sample as well as the abundance and diversity of antibiotic resistance genes present in each sample. Our preliminary analysis of the data have revealed extensive diversity among the bacterial populations in each sample, including many bacterial species that have yet to be documented in the literature in the field.

**Activity 1**

The sampling phase of this activity has been completed. In all, twelve wastewater treatment facilities were sampled at four locations in the treatment process. Each facility was sampled at three timepoints. To date, high quality DNA (sufficient for analyses of the bacterial abundance and antibiotic resistance gene abundance) has been purified from all of these samples. The resulting DNA was used to quantify the number of bacteria present in each sample, as well as multiple genes responsible for antibiotic resistance (we have focused on tetracycline resistance for the first set of analyses) and for the spread of antibiotic resistance genes. These first analyses have shown that the samples have included bacteria that bear the antibiotic resistance genes we expected to find. We have noted differences in these abundances both between facilities and throughout the treatment process. We are assessing the significance of these findings while we continue to quantify additional antibiotic resistance genes. Our sampling efforts have achieved our milestone for collecting the necessary material to analyze, and our initial quantitation efforts have kept us on pace to complete the antibiotic resistance gene abundance analysis in time for our next milestone.

**Activity 2**

Through the efforts in this activity, we plan to catalog the antibiotic resistance genes present at multiple points in the wastewater treatment process and to document their spread throughout the bacterial communities present. That analysis requires large databases of DNA sequences from as many bacteria as possible in each sample. We have created those DNA sequence database through our contracted work with Phase Genomics, using their unique technology. We have high quality sequence data from 21 samples, derived from multiple points in the wastewater treatment process that were sampled at three timepoints each. Therefore, our sampling and sequencing efforts have been completed. The next phase of the analysis is to identify the bacteria and antibiotic resistance genes present in each sample. We have identified the bacteria in the first nine samples, which comprise a complete set of timepoints and locations for one wastewater facility. Those samples have given us an in-depth view of the bacterial community structure that is present over time. We have just begun to conduct an analysis of the antibiotic resistance genes present in those samples. The remaining sample sequences are awaiting analysis that will mimic this first batch for direct comparison.

**Dissemination**

Although the sample collection, processing, and initial analysis is complete, it is too early to disseminate the findings of this study. That said, we anticipate being ready to share some of the initial findings this year. If all goes to plan, we have plans to share the some of the data at the American Society for Microbiology's annual meeting in June 2023. We also

have a rough draft of the first scientific manuscript to be submitted for publication when the analysis of the data in that paper are complete.

# Status Update Reporting

## Status Update July 1, 2022

**Date Submitted:** July 15, 2022

**Date Approved:** August 9, 2022

**Overall Update**

The goals of this project will be achieved by quantifying antibiotic resistance genes and by characterizing the spread of each resistance gene at multiple points in the wastewater treatment process from various treatment facilities around MN. The first step in this process is to collect the necessary samples and to collect from each facility multiple times over the course of several months. To date, the sample collection and initial processing (DNA extraction) is nearly complete for all wastewater treatment facilities in this study. We have begun the analysis of the bacteria present in each sample as well as the assessment of the abundance and diversity of antibiotic resistance genes in each sample.

**Activity 1**

This activity deals with the collection and quantification of antibiotic resistance genes within many samples derived from various wastewater treatment facilities throughout MN. There are two phases to this activity. The first is the sampling and DNA extraction to generate the ~180 DNA samples that will be analyzed. We are well on our way to meeting the 12/31/2022 milestone for the completion of this first phase. As of right now, we have completed sampling from a total of 8 facilities. Each of these has been sampled on three dates, and each facility was sampled at four locations within the treatment process. Three additional facilities have been sampled at two timepoints, and one has been sampled once. We plan to collect the remaining samples well ahead of the 12/31/2022 milestone. More facilities than we originally proposed were sampled in case some of the samples are not sufficient quality to proceed. In that case, we would still be left with enough samples to perform our analyses. We have conducted initial quality control analyses on the samples we've processed to ensure they are ready to move to phase 2 of this activity (antibiotic resistance gene quantitation).

**Activity 2**

This activity pertains to the in-depth analysis of the antibiotic resistance genes in specific samples over time and throughout different wastewater treatment facilities using different technologies. Our goal is to assess which treatment technologies are most efficient at removing antibiotic resistance genes and the bacteria that harbor those genes. We have partnered with Phase Genomics to do the data collection and initial analysis of these samples. To date, nine samples have been collected, processed, and sequenced by Phase Genomics (milestone 1). The resulting data is currently being analyzed to identify the bacteria and antibiotic resistance genes in those samples (milestone 2). An additional six samples have been collected and processed and are awaiting shipment to Phase Genomics. One additional sample will be collected and processed within the next month. Once that sample is ready, we will ship all remaining samples to Phase Genomics. If sequencing these samples takes the same amount of time as the first batch, we anticipate receiving the data back from Phase Genomics sometime in October. We anticipate no delays in having the antibiotic resistance gene analysis completed in time for the 6/30/2023 date proposed for milestone 2.

**Dissemination**

At this point, we are still collecting samples and data. We have just begun the analysis phase of the project. Therefore, it is too early to begin disseminating the information. We plan to complete the analyses and to disseminate our findings at the appropriate time, in accordance with our approved work plan.