2014 Project Abstract
For the Period Ending June 30, 2017

PROJECT TITLE: Triclosan impacts on wastewater treatment
PROJECT MANAGER: Timothy M. LaPara
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2014, Chp. 226, Sec. 2, Subd. 03c

APPROPRIATION AMOUNT: $380,000
AMOUNT SPENT: $363,370
AMOUNT REMAINING: $16,630

Overall Project Outcomes and Results

Laboratory-scale sequencing batch reactors were constructed and operated in triplicate at four different concentrations of triclosan (0 μg/L, 1 μg/L, 5 μg/L , and 15 μg/L). After six weeks of operation, biomass was harvested and used for 12 discrete sequencing batch reactors (SBRs) and operated them for a total of six weeks. Chemical analysis of the biomass for triclosan concentrations confirmed that the cultures were grown over the expected range of triclosan concentrations. Metagenomic DNA was extracted and purified from the biomass from these laboratory-scale cultures. Bacterial community composition was characterized by PCR of 16S rRNA genes followed by DNA sequencing using Illumina MiSeq. Results demonstrated that bacterial community composition shifted in a statistically significant fashion in response to higher triclosan doses. Direct DNA sequencing of metagenomic DNA demonstrated that only a few antibiotic resistance genes were overrepresented in the cultures with high triclosan concentrations compared to those with low triclosan concentrations. Specifically, *tet*(R) and *tet*(A) genes were overrepresented in the 5 μg/L triclosan treatment; *tet*(R), *tet*(A), *tet*(X), *msrE*, and *sul1* were overrepresented in the 15 μg/L triclosan treatments. No statistical difference was observed for more than 2,100 other antibiotic resistance genes. Metagenomic DNA was also directly cloned and introduced into *E. coli* to select for functional antibiotic resistance genes. Each of the resulting clone libraries averaged 20,000 clones, the equivalent of more than 1300 bacterial genomes. All libraries were selected for genes conferring resistance to a panel of antibiotics. Sequence analysis indicated that the enzymes encoded by the resistance genes ranged from 57 to 100 percent identical to the closest matching proteins in the GenBank database. Many of these genes were found adjacent to each other on single DNA molecules. Two clones contained multiple resistance genes on integrons in arrangements that have not been previously reported in the literature.

Project Results Use and Dissemination

Results from this project have been presented at Microbe 2016, the annual meeting of the American Society for Microbiology and at the Functional Metagenomics 2016 conference in Inderøy, Norway. Manuscripts are currently being written for publication in the peer-reviewed archival literature.

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

Date of Report: December 1, 2017

Date of Next Status Update Report: N/A

Date of Work Plan Approval: June 4, 2014

Project Completion Date: June 30, 2017

Does this submission include an amendment request? _YES_

PROJECT TITLE: Triclosan impacts on wastewater treatment

Project Manager: Timothy M. LaPara

Organization: University of Minnesota

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Location: Hennepin, Ramsey

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$380,000**
	Amount Spent:	**$363,370**
	Balance:	**$16,630**

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

Appropriation Language:
$380,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to assess the role of the commercially used antibacterial agent triclosan in creating antibiotic resistant bacteria during the municipal wastewater treatment process. This appropriation is available until June 30, 2017, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Triclosan impacts on wastewater treatment

II. PROJECT STATEMENT:

An emerging paradigm for thwarting the spread of antibiotic resistance is to enhance the nation's municipal wastewater treatment infrastructure. The rationale for this paradigm is that people taking antibiotics will select for antibiotic resistant bacteria in their gastrointestinal tracts and then release these organisms upon defecation. This fecal material then coalesces at municipal wastewater treatment facilities where the treatment process could be used to eliminate antibiotic resistant bacteria. Because spent soap, toothpaste, etc., are washed down drains, however, municipal wastewater contains high concentrations of triclosan, thus exposing the antibiotic resistant bacteria from people's gastrointestinal tracts to yet another antimicrobial agent. Triclosan could, therefore, drive the selection of multiple antibiotic resistance during the wastewater treatment process, creating new bacterial strains that are resistant to numerous antibiotics — more commonly known as "super bugs" for their ability to resist many and potentially all antibiotics. Most troubling, however, is that treated wastewater with significant quantities of superbugs could be released to Minnesota's surface waters, creating a key route by which antibiotic resistance can spread to more people.

The proposed project will provide critically important information for the State of Minnesota as it considers future legislation to ban or to restrict triclosan use within the State. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) is an antibacterial agent used in numerous commercial products, including liquid hand soap, toothpaste, cosmetics, and children's toys. Triclosan, however, has become controversial. Scientific studies have suggested numerous adverse effects, including: reduced human immune function, bioaccumulation in the environment leading to algal toxicity, and the accumulation of triclosan-derived dioxins in lake and river sediments. In contrast, the American Cleaning Institute has maintained that triclosan-containing soaps have a decades-long track record of safety and play a beneficial role in the daily hygiene routine of millions of people. Recently, the State of Minnesota considered a bill to ban triclosan.

One of the primary concerns regarding the use of triclosan is that it selects for antibiotic resistance, not just to triclosan but also to a multitude of other antibiotics. Antibiotic resistance is a pending medical catastrophe. One example of antibiotic resistance (methicillin resistant Staphylococcus aureus – MRSA) is responsible for more deaths in the United States than emphysema, HIV/AIDS, Parkinson's disease and homicide (combined). In addition, the economic cost of additional medical treatments necessitated by antibiotic resistance is estimated to be $20 to $40 billion dollars each year.

III. PROJECT STATUS UPDATES:

Project Status as of January 1, 2015:

To date, we have constructed and operated 12 discrete sequencing batch reactors (SBRs) and operated them for a total of six weeks. These SBRs were fed four different triclosan concentrations (three replicates of each concentration). Biomass samples have been collected and are awaiting analysis. Chemical analysis of the biomass for triclosan concentrations is on-going and results are anticipated soon. Metagenomic and functional metagenomic analyses will not commence until triclosan concentrations in the biomass have been determined.

Project Status as of July 1, 2015:

To date, we almost completed Activity 1, which was to operate laboratory-scale sequencing batch reactors at different triclosan concentrations. The remaining work on this activity is to measure the triclosan that was adhered to the bacterial cells. Substantial progress has been made with the Activity 2. Genomic DNA has been extracted and purified and analysis is on-going. We have obtained metagenomic DNA and analysis of this data is on-going. We have processed our samples for bacterial community analysis and we are waiting for the results to be sent to us. We are currently in the process of developing a novel technique to quantify multiple antibiotic

resistance genes simultaneously. We are also progressing towards completion of Activity 3. We have constructed fosmid libraries from three of the samples. The first two of these libraries have been subjected to antibiotics and have yielded resistant clones. Our ongoing efforts are focused on characterization of these clones, identification of clones in the third library, and construction of additional libraries.

Project Status as of January 1, 2016:

To date, we have completed Activity 1, which was to operate laboratory-scale sequencing batch reactors at different triclosan concentrations. Substantial progress has been made with the Activity 2. Genomic DNA has been extracted and purified and analysis is on-going. We have characterized the bacterial community composition by 16S rRNA gene sequence analysis. We have obtained shotgun metagenomic DNA; the analysis of this data is on-going (50% complete). We are currently in the process of developing a novel technique to quantify multiple antibiotic resistance genes simultaneously; the development of this technique is about 80% complete. The efforts associated with Activity 3 have resulted in the identification of clones that confer resistance to multiple antibiotics. All of the existing libraries have been selected for resistant clones. For each clone, the level of resistance has been determined using through minimum inhibitory concentration assays. Throughout this process, we have continued extracting DNA from the remaining samples produced in Activity 1. That genetic material is currently being used to construct additional libraries to use in identification of additional resistance genes.

Project Status as of July 1, 2016:

As stated previously, we have completed Activity 1, which was to operate laboratory-scale sequencing batch reactors at different triclosan concentrations, thereby providing the samples for analysis in Activity 2 and Activity 3. Substantial progress has been made with the Activity 2. Genomic DNA has been extracted and purified and analysis is on-going. We have characterized the bacterial community composition by 16S rRNA gene sequence analysis. We have obtained shotgun metagenomic DNA from which we have been able to estimate the quantities of several hundred antibiotic resistance genes; this data suggests that the presence of antibiotic resistance genes is not affected by the concentrations of triclosan investigated in this study. We also successfully developed a novel technique to quantify multiple antibiotic resistance genes simultaneously; we anticipate applying the method to our samples in the near future. We are currently trying to analyze the metagenomic data to determine if the presence of triclosan is associated with shifts in the profile of functional genes, which would be a collateral effect of triclosan. Substantial progress has also been made with Activity 3. Libraries have been constructed for functional metagenomic screening from samples exposed to each of the triclosan concentrations. To date, all concentrations have been used as the source material to build at least one library, and we are currently constructing libraries for replicate cultures. Every library has been archived and selected for resistance to a panel of 13 antibiotics.

Project Status as of January 1, 2017:

As stated previously, we have completed Activity 1, which was to operate laboratory-scale sequencing batch reactors at different triclosan concentrations, thereby providing the samples for analysis in Activity 2 and Activity 3. Activity 2 is nearing completion. The bacterial communities from the bioreactors have been characterized and triclosan has been shown to have a statistically significant impact. The shotgun metagenomic sequence analysis suggests that triclosan has no significant effect on more than 1000 different antibiotic resistance genes, although a difference was observed for a handful of genes. These results have been validated using a novel, microfluidic high-throughput quantitative polymerase chain reaction (qPCR) approach. We are currently trying to analyze the metagenomic data to determine if the presence of triclosan is associated with shifts in the profile of functional genes, which would be a collateral effect of triclosan. Activity 3 is nearing completion. Libraries from all samples have been constructed, screened for resistance genes, and archived.

Clones arising from the final two libraries are undergoing quality control assessment before being submitted for sequence analysis. All remaining clones have been submitted for sequencing.

Amendment Request (12/1/2017)
Because personnel costs were higher than expected and equipment/tools/supplies costs were lower than expected, $14,433 is shifted from equipment/tools/supplies to personnel.

Updated 12-15-17 Overall Project Outcomes and Results

The discovery and use of antibiotics – drugs that specifically target and kill bacterial diseases – is one of the great scientific achievements of the 20th century. Unfortunately, the use and misuse of antibiotics over the last 7 decades has led to the problem of antibiotic resistance. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether), for example, is used as an antibacterial agent in hand soap, toothpaste, cosmetics, and children's toys. Triclosan has become controversial because it is believed to lead to elevated levels of antibiotic resistance, not just to triclosan but also to a multitude of other antibiotics. Because spent soap, toothpaste, etc., are washed down drains, municipal wastewater contains high concentrations of triclosan; thus, triclosan could drive the selection of multiple antibiotic resistance during the wastewater treatment process. Most worrisome is that treated wastewater with significant quantities of superbugs could be released to Minnesota's surface waters, creating a key route by which antibiotic resistance can spread to people and to wildlife.

In this study, laboratory-scale sequencing batch reactors were constructed and operated in triplicate at four different concentrations of triclosan. Metagenomic DNA was extracted and purified from the biomass from these laboratory-scale cultures and used to determine bacterial community composition, used to determine the quantities of more than 2,100 different antibiotic resistance genes, and investigated as a source of novel antibiotic resistance genes. Bacterial community composition changed in a statistically significant fashion in response to higher triclosan doses, demonstrating the effect of triclosan on microorganisms. Higher levels of triclosan also selected higher levels of five different antibiotic resistance genes (*tet*(R), *tet*(A), *tet*(X), *msrE*, and *sul1*). Finally, the bacterial communities receiving the highest dose of triclosan contained numerous novel antibiotic resistance genes, including two integrons (known for their link to multiple antibiotic resistance) with genetic arrangements that have not been previously reported.

This research demonstrates that triclosan leads to higher levels of known and novel antibiotic resistance genes at municipal wastewater treatment facilities, which directly impact Minnesota's surface waters. This research further supports Minnesota's recent ban on the use of triclosan in liquid handsoap and in other personal care products as a means of protecting Minnesota's environment and public health. This research also suggests that the indiscriminate use of other antibacterial compounds in consumer products could also lead to higher levels of antibiotic resistance.

Overall Project Outcomes and Results:

Laboratory-scale sequencing batch reactors were constructed and operated in triplicate at four different concentrations of triclosan (0 μg/L, 1 μg/L, 5 μg/L , and 15 μg/L). After six weeks of operation, biomass was harvested and used for 12 discrete sequencing batch reactors (SBRs) and operated them for a total of six weeks. Chemical analysis of the biomass for triclosan concentrations confirmed that the cultures were grown over the expected range of triclosan concentrations. Metagenomic DNA was extracted and purified from the biomass from these laboratory-scale cultures. Bacterial community composition was characterized by PCR of 16S rRNA genes followed by DNA sequencing using Illumina MiSeq. Results demonstrated that bacterial community composition shifted in a statistically significant fashion in response to higher triclosan doses. Direct DNA sequencing of metagenomic DNA demonstrated that only a few antibiotic resistance genes were overrepresented in the cultures with high triclosan concentrations compared to those with low triclosan concentrations. Specifically, *tet*(R) and *tet*(A) genes were overrepresented in the 5 μg/L triclosan treatment; *tet*(R), *tet*(A), *tet*(X), *msrE*, and *sul1* were overrepresented in the 15 μg/L triclosan treatments. No statistical difference was observed for more than 2,100 other antibiotic resistance genes. Metagenomic DNA was also

directly cloned and introduced into *E. coli* to select for functional antibiotic resistance genes. Each of the resulting clone libraries averaged 20,000 clones, the equivalent of more than 1300 bacterial genomes. All libraries were selected for genes conferring resistance to a panel of antibiotics. Sequence analysis indicated that the enzymes encoded by the resistance genes ranged from 57 to 100 percent identical to the closest matching proteins in the GenBank database. Many of these genes were found adjacent to each other on single DNA molecules. Two clones contained multiple resistance genes on integrons in arrangements that have not been previously reported in the literature.

Amendment Request (12/1/2017)
Because personnel costs were higher than expected for Activity 2 and equipment/tools/supplies costs were lower than expected for Activities 2 and 3, $14,433 is shifted from equipment/tools/supplies to personnel.

IV. PROJECT ACTIVITIES AND OUTCOMES:
The project has been divided into three discrete activities. Activity 1 will be an experiment in which model bacterial communities will be grown at different concentrations of triclosan; these model communities are necessitated because we are unable to control triclosan concentrations at genuine wastewater treatment facilities. Activity 2 will focus on characterizing the *known* antibiotic resistance genes in the model bacterial communities grown in Activity 1; these antibiotic resistance genes will be quantified by real-time PCR and by shotgun metagenomics. Activity 3 will look for novel antibiotic resistance genes using a functional metagenomic approach.

ACTIVITY 1: Sequencing Batch Reactor operation and sample collection

Description:
This activity will involve the fabrication of model wastewater treatment bioreactors in the laboratory. These wastewater treatment bioreactors will be grown at different concentrations of triclosan, allowing us to directly test the ability to triclosan to select for antibiotic resistance genes. The outcome of Activity 1 will be bacterial biomass that will be queried for the presence of antibiotic resistance genes in Activity 2 (known genes) and Activity 3 (novel genes).

Summary Budget Information for Activity 1:

ENRTF Budget: $ 19,351
Amount Spent: $ 19,151
Balance: $ 200

Activity Completion Date:

Outcome	Completion Date	Budget
1. *Construct and Inoculate SBRs*	August 1, 2014	$14,351
2. *Collect samples*	January 1, 2015	$5,000

Activity Status as of January 1, 2015:

To date, we have constructed and operated 12 discrete sequencing batch reactors (SBRs) and operated them for a total of six weeks. These SBRs were fed four different triclosan concentrations (three replicates of each concentration). Biomass samples have been collected and are awaiting analysis. Chemical analysis of the biomass for triclosan concentrations is on-going and results are anticipated soon.

Activity Status as of July 1, 2015:

Preliminary results of triclosan concentrations have been obtained and are currently being re-analyzed to ensure the validity of the results. Once these results have been obtained, this Activity will be complete.

Activity Status as of January 1, 2016:

This activity is now complete. We have measured triclosan concentrations, confirming that the bioreactors were operated at different triclosan concentrations (ranging from no triclosan to 15 parts per billion of triclosan), as intended.

Activity Status as of July 1, 2016:

As stated previously, this activity is now complete. This activity provided the microorganisms and DNA that are analyzed, in detail, as described in Activity 2 and Activity 3.

Activity Status as of January 1, 2017:

As stated previously, this activity is now complete. This activity provided the microorganisms and DNA that are analyzed, in detail, as described in Activity 2 and Activity 3.

Final Report Summary:

This activity was successfully completed. Laboratory-scale sequencing batch reactors were constructed and operated in triplicate at four different concentrations of triclosan (0 μg/L, 1 μg/L, 5 μg/L , and 15 μg/L; Activity 1). After six weeks of operation, biomass was harvested and used for 12 discrete sequencing batch reactors (SBRs) and operated them for a total of six weeks. Chemical analysis of the biomass for triclosan concentrations confirmed that the cultures were grown over the expected range of triclosan concentrations. Biomass from these reactors was then used as source material for shotgun metagenomics (Activity 2) and qPCR to determine the effect of triclosan on antibiotic resistance development and for functional metagenomics to detect novel resistance genes (Activity 3).

ACTIVITY 2: Characterization of known antibiotic resistance genes by quantitative polymerase chain reaction and shotgun metagenomics

Description:

In this activity, the biomass grown in Activity 1 will be assayed for the quantity/presence of antibiotic resistance genes. Two different, but complementary approaches, will be used. The first approach will involve quantitative, real-time polymerase chain reactor (qPCR) to precisely quantify the amount of a handful of specific antibiotic resistance genes in this biomass. The second approach will use shotgun metagenomics to query the bacterial biomass for all known antibiotic resistance genes. The outcome of this Activity will be detailed knowledge of the precise quantities of a handful of specific antibiotic resistance genes as well as broad knowledge of the diversity of all known antibiotic resistance genes in these samples.

Summary Budget Information for Activity 2:

ENRTF Budget: $~~224,366~~ 225,692
Amount Spent: $225,499
Balance: $193

Activity Completion Date:

Outcome	Completion Date	Budget
1. *DNA extraction and purification*	March 15, 2015	$22,500
2. *Community analysis of 16S rRNA genes*	July 1, 2015	$22,500
3. *qPCR targeting antibiotic resistance genes*	October 15, 2015	~~$59,366~~ $60,499
4. *Shotgun metagenomics*	January 1, 2016	$60,000
5. *Data analysis*	January 1, 2017	$60,000

Activity Status as of January 1, 2015:

The efforts to date have focused on analyzing the biomass collected to determine the concentration of triclosan on the biomass. To date, we have prepped the biomass for solid phase extraction, purified the samples using silica columns, and attempted to quantify triclosan using high-pressure liquid chromatography. This analytical instrument, however, is not capable of detecting especially low concentration of triclosan; we are going to soon analyze these samples by liquid chromatography tandem mass spectrometry, where we anticipate being able to quantify triclosan levels. We are waiting for the quantification of triclosan levels in the SBR biomass before proceeding to extract purify the metagenomic DNA and then perform quantitative polymerase chain reaction tagerting known antibiotic resistance genes (Outcome 3) and Illumina MiSeq analysis (Outcomes 2 and 4).

Activity Status as of July 1, 2015:

Genomic DNA has been extracted, purified, and quantified from the SBRs. These samples were deemed of sufficient quality for analysis via shotgun metagenomics (Outcome 4) and we sent the University of Minnesota Genomics Center for analysis. This data has been received and analysis has commenced; due to the quantity of data (~440,000,000 DNA sequences), this data analysis will require substantial time to occur. We have amplified the 16S rRNA gene from these communities (Outcome 2) and sent the samples to the University of Minnesota Genomics Center for analysis but we have yet to receive these results. We are still in the process of developing our microarray technique for quantifying antibiotic resistance genes by qPCR (Outcome 3).

Activity Status as of January 1, 2016:

Outcome 1. This work was completed in a prior project period.
Outcome 2. We have successfully analyzed the community profiles for 16S rRNA gene sequences. We applied four different multivariate statistical tests, three of which confirmed that the different triclosan concentrations correlate to statistically different bacterial community composition.
Outcome 3. We are still in the process of developing and optimizing qPCR methods (via microarray/microfluidic device) for analysis. We have successfully optimized ~40 different methods as of this time; we should have developed 48 methods (for use one 48×48 array) by March 2016. We anticipate that actual analysis will require less than 1 week to perform, followed by a substantial amount of time for data analysis.
Outcome 4. We have made substantial progress on analyzing the shotgun metagenomic data. We have successfully assembled the sequences into contigs. We have then aligned the data with known databases of antibiotic resistance genes. Given the enormous size of the data and the number of samples, we are still trying to parse the results into meaningful metrics that can be used to discern differences between the cultures as a function of triclosan concentration.
Outcome 5. Each of Outcomes 2-4 require a substantial effort in data analysis. Progress with data analysis varies substantially; we therefore described the progress with data analysis with each Outcome.

Activity Status as of July 1, 2016:

Outcome 1. This work was completed in a prior project period.
Outcome 2. This work was completed during the previous project period.
Outcome 3. We are have developed a novel qPCR method (via microarray/microfluidic device) for analyzing more than 48 different antibiotic resistance genes. We hope to apply this method to our samples in the very near future.
Outcome 4. We have made substantial progress on analyzing the shotgun metagenomic data. We have successfully assembled the sequences into contigs. We have then aligned the data with known databases of antibiotic resistance genes. We have been able to successfully quantify and compare the quantities of antibiotic resistance genes in our sequence pools. We are currently attempting to analyze this data to determine if triclosan caused changes in the genetic functions in the bacterial community.

Outcome 5. Each of Outcomes 2-4 require a substantial effort in data analysis. Progress with data analysis varies substantially; we therefore described the progress with data analysis with each Outcome.

Activity Status as of January 1, 2017:

Outcome 1. This work was completed in a prior project period.
Outcome 2. This work was completed during the previous project period. These results demonstrated that triclosan had a statistically significant effect on bacterial community composition.
Outcome 3. We developed a novel qPCR method (via microarray/microfluidic device) for simultaneously analyzing 48 different antibiotic resistance genes. We have applied this method to our samples. This Outcome is now complete. The results suggest that triclosan has no effect on the different antibiotic resistance genes that we quantified.
Outcome 4. This Outcome is now completed. We generated more than 200,000,000 DNA sequences from which we were able to query more than 1,000 antibiotic resistance genes. Triclosan had no statistical impact on the quantity of the overwhelming majority of these antibiotic resistance genes. Triclosan did affect the quantities of a handful of genes, which could reflect a mechanistic advantage or simply random variation. We are currently attempting to reconcile these results with the existing knowledge of these genes and the known impacts of triclosan on bacterial physiology.
Outcome 5. Each of Outcomes 2-4 require a substantial effort in data analysis. We have completed the majority of data analysis, with the exception of determining the effect of triclosan on the types and quantities of functional genes in the bacterial community. This work is on-going and is anticipated to be completed by March/April 2017.

Final Report Summary:

This task was successfully completed. Metagenomic DNA was extracted and purified from the biomass from the laboratory-scale cultures. Bacterial community composition was characterized by PCR of 16S rRNA genes followed by DNA sequencing using Illumina MiSeq. Results demonstrated that bacterial community composition shifted in a statistically significant fashion in response to higher triclosan doses when analyzed by principal coordinates analysis of 3 different distance matrices (Euclidean, Unweighted UniFrac, and Bray Curtis). Direct DNA sequencing of metagenomic DNA demonstrated that only a few antibiotic resistance genes were overrepresented in the cultures with high triclosan concentrations compared to those with low triclosan concentrations. Specifically, *tet*(R) and *tet*(A) genes were overrepresented in the 5 µg/L triclosan treatment; *tet*(R), *tet*(A), *tet*(X), *msrE*, and *sul1* were overrepresented in the 15 µg/L triclosan treatments. No statistical difference was observed for more than 2,100 other antibiotic resistance genes.

ACTIVITY 3: Characterization of novel antibiotic resistance genes by functional metagenomics

Description:

In this activity, the biomass grown in Activity 1 will be assayed for the presence of novel, previously unknown antibiotic resistance genes using a functional metagenomic approach. The outcome of this Activity will be knowledge of the ability of triclosan to select for novel, previously-undiscovered antibiotic resistance genes.

Summary Budget Information for Activity 3:

ENRTF Budget: ~~$136,283~~ 134,957
Amount Spent: $118,721
Balance: $16,236

Activity Completion Date:

Outcome	Completion Date	Budget
1. *Fosmid library construction*	October 15, 2015	$26,283

2. *Screening of clones*	October 16, 2016	~~$85,000~~ $83,674
3. *DNA sequencing of clones*	January 1, 2017	$25,000

Activity Status as of January 1, 2015:

No progress has been made on this activity as of this time. We are waiting for the quantification of triclosan levels in the SBR biomass before proceeding.

Activity Status as of July 1, 2015:

The first three fosmid libraries have been constructed and stored (Outcome 1). Each library contains approximately 50,000 individual clones. Potential clones from two of these libraries that confer resistance to at least one antibiotic have been identified (Outcome 2). Currently, we are verifying that these clones contain resistance genes and are unique before submitting them for sequencing (Outcome 3). We have also begun work to quantify the level of resistance conferred by each clone.

Activity Status as of January 1, 2016:

All clones from the libraries mentioned in the previous progress report have been verified to confer resistance. The level of resistance conferred by each clone has been measured using a minimum inhibitory concentration assay. To understand the degree of co-resistance conferred, these tests include all antibiotics in our study regardless of what antibiotic was originally used to identify the clone. Three of the clones have been used in preliminary sequencing experiments in preparation for a larger scale analysis of all isolated clones (Outcome 3). Remaining samples are being processed to yield larger libraries to serve as source material for selection and identification of additional clones containing resistance genes (Outcome 2).

Activity Status as of July 1, 2016:

Every library has been selected for resistance to the antibiotics listed in the original proposal, as well as additional antibiotics with different modes of action. In total, 35 clones conferring resistance have been isolated (Outcome 2). One of these clones confers resistance to multiple antibiotics with different modes of action. We are currently conducting minimum inhibitory concentration assays on all clones to determine their levels of resistance. Preliminary sequencing results from one resistant clone has indicated the presence of a beta-lactamase that is known to confer resistance to penicillin-type antibiotics (Outcome 3). All remaining clones are being prepared for sequence analysis.

Activity Status as of January 1, 2017:

All samples, including replicates for cultures grown at all concentrations, have been used as the source material for construction of metagenomic libraries (Outcome 1). Every library has been selected for clones that confer resistance to one or more of 13 different antibiotics. To date, 40 resistant clones have been isolated and verified (Outcome 2). Approximately 10 additional clones are currently being tested to verify that they harbor true resistance genes. All clones that passed the quality control process have been submitted to the University of Minnesota Genomics Center for sequencing using their latest technology (Outcome 3). While those samples are being processed, minimum inhibitory concentration assays are being conducted on all remaining clones to measure their resistance profiles.

Final Report Summary:

All of the active clones from all of the metagenomic libraries were fully sequenced using the PacBio sequencing technology. Sequence analysis indicated that the enzymes encoded by the resistance genes ranged from 57 to

100 percent identical to the closest matching proteins in the NCBI database. Many of clones contained multiple resistance genes linked to each other on single DNA molecules. In two cases, the resistance genes were located on integrons in arrangements that have not been previously reported in the literature. MIC analyses have been conducted on all resistant clones against multiple antibiotics to link the functional resistance phenotypes to the presence of predicted resistance genes in the sequence data.

V. DISSEMINATION:

Description:

Findings will be disseminated and archived via reports to LCCMR, peer-reviewed publications, and presentations at conferences. We will also, when appropriate, disseminate results via press releases to the media. The audience is not only the scientific community, but also the public, policymakers, and practitioners. The work will also be of interest to the medical community and we will seek avenues to share the results with this community.

Activity Status as of January 1, 2015:

There has been no dissemination activity during this reporting period.

Activity Status as of July 1, 2015:

There has been no dissemination activity during this reporting period.

Activity Status as of January 1, 2016:

There has been no dissemination activity during this reporting period.

Activity Status as of July 1, 2016:

Results from Activity 2 were presented in poster form at Microbe 2016, which is the annual meeting of the American Society for Microbiology. Results from Activity 3 were presented in poster form at the national meeting of the American Chemical Society in June 2016.

Activity Status as of January 1, 2017:

Results from Activity 3 were presented in poster form at the Functional Metagenomics 2016 conference in Inderøy, Norway in September 2016.

Final Report Summary:

Preliminary results from this project have presented at two National/International Conferences. We are currently writing manuscripts for peer-reviewed publications.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Explanation
Personnel:	$ 267,771	For Drs. Donato and LaPara for directing the project; for a graduate student at the University of Minnesota; for 5 undergraduate students at the University of St. Thomas

Professional/Technical/Service Contracts:	$0	
Equipment/Tools/Supplies:	$110,729	General laboratory supplies ($5,000) DNA sequencing ($62,500) Bioreactors ($5,000) qPCR reagents ($10,000) Supplies for functional metagenomics/DNA extraction kits, cloning kits, competent cells, etc. ($28,229)
Travel Expenses in MN:	$1,500	Miscellaneous travel within MN. This will include travel to treatment facilities for bacterial inocula, travel between Universities for research duties and meetings, and travel to miscellaneous suppliers (e.g., hardware stores)
Other:	$	
TOTAL ENRTF BUDGET:	**$380,000**	

Explanation of Use of Classified Staff: N/A

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

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 2.25

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

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
University of St. Thomas	$67,500	$30,863	In-kind contribution; indirect costs not charged to this project
State			
University of Minnesota	$100,000	$91,420	In-kind contribution; indirect costs not charged to this project
TOTAL OTHER FUNDS:	**$167,500**	**$95,064**	

VII. PROJECT STRATEGY:

A. Project Partners: N/A

B. Project Impact and Long-term Strategy:

The goal of this proposed project is to understand the role of triclosan in selecting for antibiotic resistant bacteria during the municipal wastewater treatment process. This research will guide the State of Minnesota as it considers its future wastewater treatment needs; this could include limitations on the use of triclosan, improved wastewater effluent disinfection, better management of residual wastewater solids, or other initiatives to reduce the role of triclosan and municipal wastewater treatment in the spread of antibiotic resistance.

C. Spending History: N/A

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. VISUAL ELEMENT or MAP(S): see attached visual

X. ACQUISITION/RESTORATION REQUIREMENTS WORKSHEET: N/A

XI. RESEARCH ADDENDUM: See attached

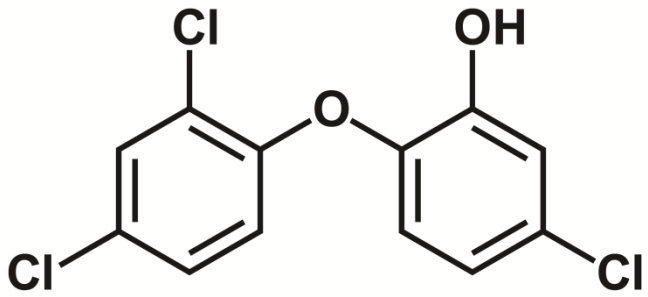
XII. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted no later than January 1, 2015, July 1, 2015, January 1, 2016, July 1, 2016, and January 1, 2017. A final report and associated products will be submitted between June 30 and August 15, 2017.

Environment and Natural Resources Trust Fund

M.L. 2014 Project Budget

Project Title: *Triclosan impacts on wastewater treatment*
Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03c
Project Manager: *Timothy M. LaPara*
Organization: *University of Minnesota*
M.L. 2014 ENRTF Appropriation: $ *380,000*
Project Length and Completion Date: *3 Years, June 30, 2017*
Date of Report: *January 12, 2017*

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Revised Activity 2 Budget 12/1/2017	Amount Spent	Activity 2 Balance	Activity 3 Budget	Revised Activity 3 Budget 12/1/2017	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***SBR operation and sample collection***			***Characterization of antibiotic resistance genes***				***Functional Metagenomics***					
Personnel (Wages and Benefits)	$13,851	$13,851	$0	~~$157,037~~	$171,470	$171,470	$0	$96,883	$96,883	$91,161	$5,722	$282,204	$5,722
Project Management, Timothy LaPara ($47,666;9.6% of time; 75% to salary, 25% to fringe benefits)													
Co-Project Manager, Justin Donato ($30,851; 11.5% of time; 75% to salary, 25% to fringe benefits)													
Graduate Student at U of M ($123,222; 50% of time; 50% to salary, 50% to benefits)													
Undergraduate Students at UST ($66,032; 5 students; paid hourly; 100% to salary)													
Equipment/Tools/Supplies *(General laboratory supplies ($5,000), DNA sequencing ($62,500), Bioreactors ($5,000), qPCR reagents ($10,000), Supplies for functional metagenomics/DNA extraction kits, cloning kits, competent cells, etc. ($28,229))*	$5,000	$5,000	$0	~~$66,829~~	$53,722	$53,722	$0	~~$38,900~~	$37,574	$27,560	$10,014	$96,296	$10,014
Travel expenses in Minnesota *(Miscellaneous travel to get samples and supplies; for vehicle rental or reimbursement of personal vehicles.)*	$500	$300	$200	$500	$500	$307	$193	$500	$500	$0	$500	$1,500	$893
COLUMN TOTAL	**$19,351**	**$19,151**	**$200**	**~~$224,366~~**	**$225,692**	**$225,499**	**$193**	**~~$136,283~~**	**$134,957**	**$118,721**	**$16,236**	**$380,000**	**$16,629**



Triclosan

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Municipal Wastewater Treatment

Antibiotic Resistance

[*tet*(R), *tet*(A), *tet*(X), *msrE*, *sul1*]