M.L. 2017 Project Abstract
For the Period Ending June 30, 2021

PROJECT TITLE: Mapping Taxonomy and Environmental Toxicology of Minnesota Freshwater Sponges
PROJECT MANAGER: Anthony Schroeder
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WEBSITE: https://freshwatersponges.crk.umn.edu
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2017, Chp. 96, Sec. 2, Subd. 03m as extended by M.L. 2020, First Special Session, Chp. 4, Sec. 2

APPROPRIATION AMOUNT: $258,000
AMOUNT SPENT: $250,305
AMOUNT REMAINING: $7,695

Sound bite of Project Outcomes and Results

Our project identified freshwater sponges are widely distributed throughout Minnesota's lakes and rivers. Sponges are thought to be bio-indicators of good water quality, suggesting many rivers and lakes in Minnesota are of relatively good quality. We identified new species of freshwater sponges not described previously, so there is likely significant amounts of biological diversity not described in the state. As filter feeders, it doesn't appear that freshwater sponges are accumulating pollutants that can be passed through the food chain.

Overall Project Outcome and Results

Freshwater sponges are the simplest animals and play a vital role in the aquatic ecosystem by functioning as a filter feeder and providing habitat and nutrients for other aquatic life. As filter feeders, freshwater sponges could potentially accumulate pollutants and transfer them through the food chain to game fish and other economically important aquatic and terrestrial organisms. Furthermore, despite their importance, information on the distribution of freshwater sponges in Minnesota lakes and rivers is very limited. The primary goals of this project were to **(1) determine the diversity and distribution of freshwater sponges in Minnesota's water basins and watersheds and to (2) determine if these freshwater sponges are accumulating toxic pollutants.**

From our sampling of freshwater sponges, we found freshwater sponges are widely distributed throughout the state of Minnesota. We sampled over one hundred locations and found freshwater sponges at over 75% of the locations sampled, resulting in a total of 169 individual freshwater sponges collected. The majority of the freshwater sponges collected are species that have previously been identified in the state. We identified one new species of freshwater sponge from this project, while potentially identifying a few more after additional follow-up analyses.

From the chemical analysis of collected sponges there does not appear to be an accumulation of pollutants within the sponge that could be passed through the food chain. Our chemical analysis did identify interesting and unique chemical compounds in the freshwater sponges that has the potential for having bioactivity and could be used for human purposes.

The results of this project showed that freshwater sponges are widely distributed in the state of Minnesota, supporting the notion that these animals are important for the freshwater ecosystem. We have identified new species of freshwater sponges, and importantly, it doesn't appear that sponges are accumulating pollutants that could remain in the ecosystem. We were also able to train 18 undergraduate students in biological and

chemical research. Many of these students have gone on to be scientists, nurses, doctors and other important jobs in Minnesota.

Project Results Use and Dissemination

The dissemination of the project has occurred through multiple mediums. This project was highlighted in the Minnesota DNR's Minnesota Conservation Volunteer Magazine. The project has also been shared with the general public by being added to the Minnesota State Parks and Trails Geocaching Aquatic Quest. An important aspect of this project was providing research opportunities for our undergraduate students at the University of Minnesota Crookston. The research involvement by students was highlighted in a number of publications. This results of this project were also presented at multiple scientific and non-scientific conferences by faculty and students.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2017 LCCMR Work Plan Final Report

Date of Submission: August 16, 2021

Date of Next Status Update Report: Final Report

Date of Work Plan Approval: 06/07/2017

Project Completion Date: June 30, 2021

PROJECT TITLE: Mapping Taxonomy and Environmental Toxicology of Minnesota Freshwater Sponges

Project Manager: Anthony Schroeder

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Location: Statewide

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$258,000**
	Amount Spent:	**$250,305**
	Balance:	**$7,695**

Legal Citation: M.L. 2017, Chp. 96, Sec. 2, Subd. 03m as extended by M.L. 2020, First Special Session, Chp. 4, Sec. 2

Appropriation Language:
$258,000 the first year is from the trust fund to the Board of Regents of the University of Minnesota, Crookston, to determine freshwater sponge distribution, identify and quantify accumulated contaminants, and provide educational research opportunities to undergraduate students. This appropriation is available until June 30, 2020, by which time the project must be completed and final products delivered.

M.L. 2020 - Sec. 2. ENVIRONMENT AND NATURAL RESOURCES TRUST FUND; EXTENSIONS. [to June 30, 2021]

I. PROJECT TITLE: Minnesota's Freshwater Sponges: Mapping Taxonomy and Environmental Toxicology

II. PROJECT STATEMENT:

Sponges are the simplest forms of animal life and play a vital role in the aquatic ecosystem by providing habitat and nutrients for other aquatic life. Most of the sponges that have been studied are found in the marine environment and are known to accumulate pollutants similar to those found in MN waters (e.g., polyaromatic hydrocarbons or PAHs). Due to their similar structure, freshwater sponges are likely accumulating these toxic substances found in MN waters and may be transferring them through the food chain to game fish and other economically important aquatic and terrestrial organisms. Despite their importance in aquatic ecosystems, information on distribution of freshwater sponges in MN lakes and rivers is very limited. The primary goals of this project are to **(1) determine the diversity and distribution of freshwater sponges in Minnesota's water basins and watersheds and to (2) determine if these freshwater sponges are accumulating toxic pollutants.** The proposed study will generate novel and critically important information regarding distribution of freshwater sponges and bioaccumulation, aiding in the fight to protect Minnesota's aquatic ecosystems.

We will collect and identify sponges from two lakes and/or rivers in each of the ten watersheds in MN (Cedar River, Des Moines River, Lake Superior, Lower Mississippi River, Minnesota River, Missouri River, Rainy River, Red River of the North, St. Croix River, Upper Mississippi River Basins) and map their geographic distribution. Taxonomic identification will be performed using morphological and molecular analyses. Subsequent to the identification of sponges, chemical analyses will be performed to identify and quantify accumulating pollutants. Chemical analyses will be carried out by extraction of organic compounds from the sponges and screening for the presence of pollutants using sophisticated chromatographic methods such as gas and liquid chromatography coupled with mass spectrometry (GC-MS and LC-MS).

Another goal of this project is to **(3) further strengthen interdisciplinary research among faculty at University of Minnesota Crookston (UMC) and provide its students with practical skills that could be translated into their careers**. UMC is uniquely poised to tackle this project because of its faculty and student profile. UMC faculty are recognized for their expertise in natural resources and in the natural sciences. The associated faculty have established productive interdisciplinary collaborations involving undergraduate students with varying academic backgrounds.

III. OVERALL PROJECT STATUS UPDATES:

Amendment Request (Amendment Approved by LCCMR 09/25/2017):

An amendment is being made to the work plan and budget to allow funds for an ANDI certified diver with solo diver and diver first aid certification. The certified diver will present on all collection trips in which diver for freshwater sponges occurs. The presence of a safety diver is recommended by dive professional in all recreation and commercial dive operations, it is also a safety requirement by the University of Minnesota. The certified diver will only serve in a dive safety capacity and will not perform collections, but is necessary to make sure the up most safety of the diver performing the collections. To support this position, $4,500 will be moved from the $12,000 that are currently unallocated. The position will be a competitive process that will be performed through phone interviews and does not been to have a formal request for proposal due to the amount of funds listed for the position.

Project Status as of February 1, 2018:
Between late July and early October 2017, we were able to collect freshwater sponges from lakes and rivers in Minnesota by either wading or diving. We have collected at least one sponge sample from a lake and river in three of the ten watersheds. The watersheds that we have sampled in are the Upper Mississippi River, Red River of the North, and St. Croix River. Overall, we have collected 19 individual sponge samples that are being used for morphological, molecular, and chemical analyses. The morphological preparation and analysis of each of the 19 sponges has been completed. The molecular analysis using mitochondrial and nuclear genes is ongoing for the 19 samples. The preparation of chemical extracts from some of the collected sponges has started and is also ongoing. One of the unforeseen positive outcomes for this project, thus far, has been the involvement with the general public of Minnesota. Through various methods of dissemination of this project, we have received input and feedback from over 30 individuals. These individuals have provided information related to freshwater sponge locations in Minnesota, sought more information related to sponge identification, and in many cases, expressed their interest in the project as a whole.

Project Status as of August 1, 2018:
During the spring and summer of 2018, freshwater sponge samples have been collected from additional rivers in Minnesota. We have completed our river collections in 5 of the 10 watersheds (Minnesota River, Rainy River, Red River of the North, St. Croix River, Upper Mississippi River). Thus far in 2018, we have collected an additional 21 sponge samples. Collections will continue through the fall, with the plan to complete all river sampling in each of the ten watersheds by this time. The morphological preparation for the sponges collected in 2018 are currently being processed to examine their spicules. Clean DNA sequences for the mitochondria Cytochrome Oxidase I (COX 1 or COI) has been completed for nine of the sponges collected in 2017. All collected sponges from 2017 have nuclear rRNA fragments amplified and sequencing in the works. It is expected that all the sequencing for samples collected in 2017, along with species analysis, will be completed this fall. Chemical extractions have been performed on all collected sponges from 2017. Each sponge sample has extracts prepared in methanol, dichloromethane, and hexane. Each of these extracts from 3 different sponges (9 extracts total) collected in 2017 have been analyzed by NMR and liquid chromatography analysis. The training of undergraduate students, incorporation into the Biology Program curriculum and public outreach continues to be ongoing. Five undergraduate students were involved in the sponge project during the spring semester. During the summer, three undergraduates have been involved in the project full-time. Aspects of the sponge project were incorporated into the Molecular Biology Techniques course. Additional information about the location of sponges throughout the state have been received by the citizens, dive shops, and resorts in Minnesota. We have continued to actively participate in outreach to the general public, state agencies, and others as the project is ongoing.

Project Status as of February 1, 2019:
At a little over the halfway point of the project, overall, we have made excellent progress in achieving the outcomes in each of the three activities. We haven't been able to meet the desired completion dates which we originally thought and listed, but this is expected, as this study involves a significant amount of sample collections which are subjected to and influenced by weather. For example, in activity 1, we have completed all of our river collections in all of the watersheds except for those in southern Minnesota (near the Iowa border and stretching from Wisconsin to South Dakota). We were planning on completing them in the fall of 2018, but it was an unusually wet summer and fall in southern Minnesota, where more than double the amount of average rainfall was experienced. This made the planned sampling of rivers in the area impossible because of the high river levels. These areas will be completed right away in the early spring. We have also completed some of our sampling in the lakes throughout Minnesota, but this will be now be the focus for the upcoming summer.

For all of the freshwater sponge samples collected, we have completed all of the morphological identification through spicule analysis and genetic analysis through DNA sequencing. We have now started the evolutionary analysis using the DNA sequences we obtained with other publically available DNA sequences for sponges to provide better resolution of the species of sponge we collected and the likelihood that some may be new species of sponge. We plan to begin mapping the locations of the sponges starting this spring and will continue this as we complete our collections this summer. This should provide us plenty of time to finish the mapping by June 2020.

For activity 2, all of the collected sponges from 2018 have been freeze-dried and are ready for chemical extraction. The extractions from these sponges will continue this spring. Many of the extractions for sponges collected in 2017 have been analyzed by chemical instrumentation and any remaining samples from 2017 will be completed this spring. We are possibly identifying chemicals that have not been previously reported in sponges. We have the methods in place to be able to complete the chemical analyses for the collected sponges from 2018 and this upcoming summer to complete the analyses by the June 2020 completion date.

For activity 3, we have continued to focus on the involvement and training of undergraduate students through this project. We have continued to find ways to incorporate aspects of the freshwater sponge into the curriculum at UMC. Aspects of the project were incorporated into the Biology Program's Nature of Life course again this fall semester and the Undergraduate Research in Chemistry course. This spring, we have also incorporated aspects of the sponge project into a special topics course taught by Drs. Schroeder and Dudley. The incorporation of the sponge project into these courses has allowed for fifteen additional students to be exposed to freshwater sponges and performing research related to this project. Also, the undergraduates who have worked full-time on the project during the summer have an abstract that was accepted at the National Conference for Undergraduate Research (NCUR) and they will be presenting their results from the freshwater sponge project in April.

Lastly, we have continued to focus on our outreach throughout the state of Minnesota. We are currently working with faculty at Anoka-Ramsey Community College to help them understand where freshwater sponges are found and how they can be incorporated into their curriculum. We are also working with people at the Martin Soil and Water Conservation District to help them determine if they have found sponges in CREP wetlands they sampled this summer. We have been in contact with people from the Minnesota Pollution Control Agency about the project. They have provided us with some historical information about where they have found sponges during their previous sampling. As mentioned previously, we have made considerable effort to make sure we are participating in outreach to the general public, state agencies, and others. This will continue to emphasize this aspect of the project as this allows for our project to support all of Minnesota and to make sure the project is well disseminated to all stakeholders.

Project Status as of August 1, 2019:

We have completed the sampling in at least two rivers in each of the ten major watersheds of Minnesota. We have also sampled two lakes in most of the watersheds. This will be completed yet this summer and continuing into early fall, with much of the time dedicated to diving in lakes. Early summer 2019 dive exploration revealed that sponges were not prevalent in previously reported areas. New reports from local dive shops have suggested new locations that will be explored during late summer through fall. Our sampling through the spring and summer has, thus far, provided us with 42 additional sponge samples from locations throughout Minnesota. Interestingly, we sampled 8 rivers in the northeast Lake Superior watershed, but did not find any sponges to date. Similarly, we

sampled the major rivers in southern Minnesota and did not find any sponges. It has once again been relatively wet summer in southern Minnesota, with many of the rivers higher than usual. We plan to go back to sample major river in the southern Minnesota watershed later this fall. We are on track to readily complete all collections that we mentioned in activity 1 of the proposal, with additional sampling that will be conducted if we have time. Also, all samples have been archived at the University of Minnesota Crookston, with the intention that they will be provided to the Bell Museum before the end of the project.

The 42 samples collected during the spring and summer have been separated for genetic, morphological and chemical analyses. We have completed the spicule preparations for all 42 sponges to examine the morphology. In the upcoming fall, spicules from each sample will be photographed and measured for starting species analysis. 18 of the 42 sponges collected this spring/summer has DNA extracted. The DNA has been used for amplifying the COI and 18s genes using PCR. The DNA from the other samples will be extracted and also used for amplifying these genes. The gene products will all be sequenced this coming fall and the evolutionary analysis, which has already begun, will be completed for all the sponges collected.

As mentioned, the newly collected sponges have been separated for their different analyses. Thus far, six sponge samples collected and separated for chemical analyses has been freeze-dried. These rest of the samples will be freeze-dried before the end of the summer and will be ready for chemical extraction at the beginning of the fall.

The majority of the sponges collected and freeze-dried during the summer of 2018 have been extracted using dichloromethane and methanol. The prepared extracts will be examined with GC-MS and LC-MS to identify potential contaminants. This will be completed in early in the fall. The rest of the 2018 and summer 2019 sponges will be extracted early in the fall and the chemicals will be examined using similar chemical instrumentation. The rest of the upcoming year will focus on identifying chemicals present within the sponges.

Lastly, water samples collected from each of the locations where sponges have been found has been prepared for organic, inorganic, and metal analyses. This provides us a snapshot of what the aquatic environment looks like where sponges are found. The prepared samples now just beginning to be run through LC-MS/MS and ICP-MS to analyze the water chemistry. The rest of the upcoming year will also focus on examining the water chemistry for the various lakes and rivers that sponges have been found.

Project Status as of February 1, 2020:
We are well positioned to complete all of the major goals and outcomes of this proposal by the June 30th project completion date. Almost all of activity 1 is complete. Over the course of the project, we collected 104 individual freshwater sponges across 40 lakes and rivers throughout Minnesota. All the proposed spicule morphology preparations are completed and the last of the morphological analyses is finishing up. We were able process all of these samples even quicker than expected, so we were also able to use the spicule preparations for potentially new species for scanning electron microscopy as well (see activity 1 report below). All of collected sponges have the DNA extracted from them and their DNA has been sequenced. There are a few samples that we are cleaning up the DNA further to get cleaner sequence results, but we are finished with the sequencing of the majority of the collected sponges. The geographic mapping of the sponge locations is occurring. We have an undergraduate student majoring in Natural Resources helping to map the sponge locations using GIS. It is expected the sponge map, with chemistry data incorporated with the map, will be completed by June. Lastly, each of these sponges are prepped to send to the Bell Museum for archiving.

For activity 2, extracts have been prepared for the majority of the sponges collected. There are still a few samples left to finish, but they are being worked on. It is expected the extracts from the last sponges will be completed in the next couple months, and then those extracts will be examined through GC- and LC-MS. The chemical extracts from water samples where sponges were collected are also being wrapped up. The water samples are being processed through solid phase extraction to prepare the extracts. The extracts are then being analyzed using GC- and LC-MS. We are on track to prepare and analyze the last of the water extracts before the completion of the project in June. All of the basic physiochemical and total organic carbon analyses for each of the collected water samples has been completed.

For activity 3, we have continued to have undergraduate students involved in the project. We have had three students continue to work on the project since the last report. Also since the last report, we have also had two students graduate who were working on the project. We have two more new students working on the project to help finish up the last parts of the project. We have also continued to help and support other investigators at other institutions who are performing freshwater sponge research. We are also continuing to disseminate the results of the project to our campus and to other stakeholders (see activity 3 status for details).

Project extended to June 30, 2021 by LCCMR 6/18/20 as a result of M.L. 2020, First Special Session, Chp. 4, Sec. 2, legislative extension criteria being met.

Project Status as of August 1, 2020:
At the time of our last report, we mentioned that we were well positioned to complete all of the major goals and outcomes of the project. However, one month later, the lab was shut down due to the COVID-19 pandemic. Fortunately, receiving the legislative extension for the project will provide us an opportunity see all the goals and outcomes to completion. We are just getting the lab opened up again, under socially distanced restrictions, to where we can start working on completing the project.

As mentioned in the last report, the major outcomes of activity 1 for the project are mostly completed. We were working to get the last images of the sponge spicules and also getting scanning electron microscopy images of the spicules. This was all put on hold when the lab shutdown and has yet to be restarted. As we continue to open the lab, the spicule imaging will continue and is expected to be completed this fall. We were also working with an undergraduate student in the Natural Resources Department to develop a GIS map of the locations were we sampled for freshwater sponges. This work continued even after the lab shutdown and the student has completed GIS maps for us. This will be updated with additional information as the lab reopens. Because restrictions associated with social distance and the lab being closed, we decided to use the opportunity to perform additional collections during the summer of 2020. This allowed us to perform more thorough sampling of locations where we either hadn't found sponges or found a limited number of sponges. This will provide us a larger dataset for determining the physiochemical properties of rivers and lakes needed for sponges to be present. The sponges we collect this summer will still have the spicules processed for morphology and DNA extracted for species identification. However, we have prioritized completing all of the imaging from the samples from previous years, before completing the rest of the analyses for samples collected this summer.

At our last report for activity 2, we were focusing on preparing the extracts from the last freshwater sponges that had been collected. This, too, came to a halt due to the shutting down of the lab. We have yet to finish the last extracts, but this will completed as the lab reopens. Not all of the water samples have been prepped through solid phase extraction, but this will also continue as the lab reopens. Water samples from all sampling

locations have been sent to the water analysis lab at the University of Minnesota for ICP-OES analysis. This will provided us with the presence or absence of 27 different elements and their concentrations for each location.

The shutting down of the lab from March through July, severely hampered the outcomes of activity 3. Although the project was supposed to end June 30, we had three new undergraduates who were going to start working on the project from April through the end of June. This would have provided additional research opportunities for these students. Due to the one year extension, we are fortunate enough now to have these students participate in research this upcoming fall as the lab reopens fully. Despite the lab shutting down, we did continue to communicate and work with investigators at other institutions who are performing freshwater sponge research. Sponge samples were sent to an investigator at Crown College, MN, who will be using Minnesota freshwater sponges for barcoding in her class this upcoming fall.

Amendment Request (Amendment Approved by LCCMR 03/15/2021):
An amendment is being made to the budget to move the funds from the $7500 unallocated funds budget line (line 34 in the budget) to other budget lines. $4500 will be moved to the Personnel (Wages and Benefits) budget line (line 12 in the budget). The funds are being requested to move to this budget line to be able to pay the additional undergraduates who are working on the project following the extension of the project due to COVID. The remaining $3000 in unallocated funds is requested to be moved to the General chromatographic and spectrophotometric supplies budget line (line 26 of the budget under Equipment/Tools/Supplies). These funds are requested to move to this line to purchase the last items needed to finish up the chemistry work related to Activity 2 in the proposal. All of the unallocated funds that will be moved to these lines will support the personnel and supplies needed to complete activity 2 and so the unallocated funds will move to the Activity 2 budget.

Project Status as of February 1, 2021:
Since our last report, we have been able to get closer to returning the lab to all its full capacity, while adhering to all COVID restrictions. We are now able to have more students helping on the project than earlier this fall. We feel we are in a good place to finish up the last of the outcomes needed to fully complete the project by the June 30 end date.

The majority of outcomes for activity 1 are completed; we are mostly working on the analyses for sponges collected this summer. In the proposal, we had proposed to sample two rivers and lakes in each of the 10 watersheds in Minnesota. We were able to complete this with ease. We found sponges in rivers sampled in each watershed except for the Rock River and Des Moines River watersheds, despite heavy sampling in the summer of 2019 and 2020. The greatest number of sponges were collected from rivers in the Upper Mississippi River watershed. There were fewer sponges that were found in the lakes sampled in all of the watersheds. Lake Vermillion had the greatest number of sponges collected of each of the lakes sampled. This summer we also performed additional scuba diving in lakes for sponges with mixed results of being able to collect sponges. All of the sponges that were collected this summer have been processed for spicule analysis. We still have not been able to complete the scanning electron microscopy images of spicules, due to COVID restrictions. However, the light microscopy images of the spicules using differential inference contrast is continuing and we will starting the imaging for the sponges collected this summer soon. The DNA from the sponges collected this summer has been extracted. We are working on the PCR and sequencing for cytochrome oxidase and 18s rRNA genes to determine

the species of sponges collected this summer. We had previously had a map of all collected sponge locations, but we are now updating the map to include the locations from this summer.
The majority of our focus and student personnel have been working on finishing up the outcomes for activity 2. We are working to complete the chemical extracts from the collected sponges. The extracts will be analyzed using GC-MS and LC-MS to identify contaminants present in the sponges. The water analyses for locations were the sponges were collected this summer are ongoing. This includes total organic carbon, ion concentrations analyzed by ion chromatography, and ICP, and chemical analysis using GC- and LC-MS.

For activity 3, we have been able to have undergraduate students working on the project again, with COVID restrictions in place. This spring semester, we have three new undergraduate students working on the project. One student is interested in microscopy and is working on completing the spicule imaging. The other two are working on the chemical analysis for activity 2. The one year extension has helped to provide these students with research opportunities that they may not have previously had available. Furthermore, they are developing skills in areas of their scientific interests. We still have two other students who have worked on the project previously, continuing to work with us. As mentioned in the August update, we sent sponge samples to an investigator at Crown College, MN, who will be using Minnesota freshwater sponges for barcoding in her class this upcoming fall. She used the sponges for barcoding in her class and mentioned that the students found using the sponges in her class as a valuable experience. We are sending additional samples to her for this spring semester to use in her class again. The investigator also mentioned that she has a student who would like to learn more about sponges and participate in the collect of sponges. We are planning to have the student from Crown College join us for a trip to collect sponges this summer. We have also had an abstract accepted to present our sponge research at the virtual University of Minnesota Extension Gathering Partners Conference this summer. Lastly, we have had an abstract accepted to allow us to have a research booth at the Driven 2 Discover building at the Minnesota State Fair in 2021. If the fair is held, we will present our research to the general public at the fair and allow them to learn more about sponges in the state.

Overall Project Outcomes and Results:

Freshwater sponges are the simplest animals and play a vital role in the aquatic ecosystem by functioning as a filter feeder and providing habitat and nutrients for other aquatic life. As filter feeders, freshwater sponges could potentially accumulate pollutants and transfer them through the food chain to game fish and other economically important aquatic and terrestrial organisms. Furthermore, despite their importance, information on the distribution of freshwater sponges in Minnesota lakes and rivers is very limited. The primary goals of this project were to **(1) determine the diversity and distribution of freshwater sponges in Minnesota's water basins and watersheds and to (2) determine if these freshwater sponges are accumulating toxic pollutants.**

From our sampling of freshwater sponges, we found freshwater sponges are widely distributed throughout the state of Minnesota. We sampled over one hundred locations and found freshwater sponges at over 75% of the locations sampled, resulting in a total of 169 individual freshwater sponges collected. The majority of the freshwater sponges collected are species that have previously been identified in the state. We identified one new species of freshwater sponge from this project, while potentially identifying a few more after additional follow-up analyses.

From the chemical analysis of collected sponges there does not appear to be an accumulation of pollutants within the sponge that could be passed through the food chain. Our chemical analysis did identify

interesting and unique chemical compounds in the freshwater sponges that has the potential for having bioactivity and could be used for human purposes.

The results of this project showed that freshwater sponges are widely distributed in the state of Minnesota, supporting the notion that these animals are important for the freshwater ecosystem. We have identified new species of freshwater sponges, and importantly, it doesn't appear that sponges are accumulating pollutants that could remain in the ecosystem. We were also able to train 18 undergraduate students in biological and chemical research. Many of these students have gone on to be scientists, nurses, doctors and other important jobs in Minnesota.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collection and taxonomic identification of freshwater sponges sampled from the ten major basins and watersheds in Minnesota

Description:

Freshwater sponges will be collected from two lakes and or rivers from each of the ten watersheds during the summer months (June to October) of 2017 and 2018. To provide information about freshwater habitat selection in Minnesota waters, sponges will be collected at depths ranging from 0.15 to 12 meters by two methods: 1) wading into shallow water and examining appropriate substrate (e.g., rocks, sunken trees); 2) SCUBA diving into deeper areas and examining appropriate substrate. For each sampling location the GIS coordinates, data pertaining to the body of water, the physical, chemical, and biological conditions will be obtained. ArcGIS will be used to map the physical location with various conditions where the sponges were found. These data will be used to provide the first map of where freshwater sponges are located in Minnesota.

Collected sponges from each location will be separated for genetic and morphological analyses and for chemical analysis. A sub-sample of sponge will be placed in 70% ethanol, and stored at -20^{o}C until used for genetic and morphological analyses (Activity 1). The remaining wet sponge material will be freeze-dried, dry weight of each sponge specimen recorded and used for chemical analysis (Activity 2).

A sub-sample of sponge (less than 1 cm^3) will be digested in boiling concentrated nitric acid (2 mL) for 10-15 minutes in order to retain only spicules and gemmules. The digested samples will be rinsed and prepared for initial light microscopy analysis as well as subsequent scanning electron microscope analysis. Species will be distinguished by identifying morphotraits which include examination of megascleres, microscleres, and gemmular theca.

Another sub-sample of sponge will be used for molecular identification. Nuclear and mitochondrial DNA will be extracted using commercially available extraction kits. Polymerase chain reaction (PCR) will be used with primers previously designed to amplify 18s rDNA and cytochrome oxidase I (COX 1 or COI) using cycling protocols previously published for each primer set. Amplified nuclear and mitochondrial DNA for each sponge collected will be sequenced using BigDye™ Terminator v3.1 and an ABI PRISM™ 3100 Genetic Analyzer to obtain nucleotide sequences. Nucleotide sequences will be subjected to a BLASTN search using the National Center for Biotechnology Information (NCBI) database. BLASTN results will be used to identify the species of the collected sponges.

The data obtained from sequencing the collected sponges will be used for determining phylogenetic relationships to identify potentially new freshwater sponge species. Other DNA sequences for COI and 18s rDNA

from other freshwater sponge species that have been deposited in NCBI's GenBank will be extracted. Sequences for each gene will be aligned using BioEdit v7.2 software. Aligned sequences for each gene will be used for phylogenetic analysis using maximum parsimony implemented in PAUP* v4.0 and Bayesian inference implemented in MrBayes v3.2. For the Bayesian inference, ModelTest v3.7 will be used to determine the best of fit of the data for phylogenetic analyses. When these analyses are complete we will be able to further document which species of sponges are present in Minnesota's lakes and rivers and potentially identify new species of freshwater sponges.

Summary Budget Information for Activity 1:

ENRTF Budget:	**$ 115,500**
Amount Spent:	**$ 115,500**
Balance:	**$ 0**

Outcome	**Completion Date**
1. *Collection of sponges from two lakes/rivers within each major basin/watershed in MN*	October 2018
2. *Morphological and molecular identification of collected sponges*	January 2019
3. *Geographic mapping of the distribution of freshwater sponges in MN*	June 2020

Activity 1 Status as of February 1, 2018:
Karl Anderson has collected 19 freshwater sponges from lakes and rivers from of 3 of the 10 major watersheds in Minnesota. GPS coordinates were also collected at each location where a sponge was found. The GPS coordinates will be used for geographic mapping of the distribution of freshwater sponges in the future, but this analysis has not started yet. Water/environment characteristics were recorded at each location, and water was collected and returned to the University of Minnesota Crookston for further laboratory analysis. The collected water samples have been frozen and the chemical analyses of these water samples will start this spring.

The Schroeder lab, with the assistance of three undergraduate students, has completed the morphological analysis for all 19 samples. A subsample from each sponge was boiled in nitric acid to digest the sponge and examine the structure of the spicules. Photographs have been taken of the spicules from each sample collected.

Schroeder and the undergraduate students have isolated mitochondrial and nuclear DNA from each of the 19 freshwater sponge samples. The mitochondrial DNA has been amplified by polymerase chain reaction using cytochrome oxidase I (COX1 or COI) primers for all 19 sponge samples. Amplification of the nuclear DNA using 18s rRNA primers has yet to be completed. Of the 19 samples we have obtained DNA sequences of COI for 9 of the samples. Four of the samples had DNA sequences that were clean and we could use to identify likely species using BLAST search. For these samples, two sponge samples had 99% identity to *Spongilla lacustris* and the other two had 99% identity to *Ephydatia mulleri.* The other five samples, provided DNA sequences that had background DNA contamination, so getting a clean sequence for species analysis is not possible yet. The extracted DNA from these samples are being cleaned up again and will be re-sequenced to obtain a better sequence. After good COI sequences have been obtained for these nine samples, the other ten samples will be sequenced to obtain the COI sequence for these samples.

Activity 1 Status as of August 1, 2018:

Karl Anderson with the help of undergraduate students has collected an additional 21 freshwater sponges from rivers from 5 of the 10 watersheds in Minnesota. GPS coordinates were also collected at each location where each sponge was found. The GPS coordinates were added to the where previous sponges were found and will be used for geographic mapping of the distribution of freshwater sponges in the future. Water/environment characteristics were recorded at each location, and water was collected and returned to the University of Minnesota Crookston for further laboratory analysis. The collected water samples have been frozen and the chemical analyses of these water samples will start this spring. We had planned to complete all collections in the five watersheds in southern Minnesota (along Iowa and South Dakota borders) in June. However, due to high rainfalls, the rivers in these area have been higher making it impossible to wade to collect in these areas. Sponges will be collected in these watersheds in August and September to complete the sampling of rivers in all ten watersheds. Diving in lakes will also continue August through October. It is expected that diving will occur in three more watersheds to complete diving in six of the ten watersheds.

The Schroeder lab with the help of two undergraduate students during the spring semester and two full-time students during the summer have been working on the morphological and molecular analysis of the sponges. The morphological analysis of the 21 samples collected in 2018 is ongoing. Almost half of the samples have been processed for analyzing the spicules. The rest of the spicule preparation and analysis will be completed this fall.

The molecular analyses are ongoing by the Schroeder lab with the help of the undergraduate students. All of the sponge samples from 2017 have been sequenced for the mitochondria gene Cytochrome Oxidase I (COX1 or COI). Freshwater sponges are animals with many organisms living within and among the sponges, so we have at times not been able to get clean sequences just from sponges. However, we were able to get clean sequences for another 5 samples. After performing a BLAST search of the DNA sequence for the five sponges, two more samples were identified as *Spongilla lacustris* and the other two had as *Ephydatia mulleri.* These two species of sponges have been identified from other locations collected during 2017. The fifth sample was identified as *Ephydatia fluviatilis.* This is a common freshwater sponge species that is found throughout the world, but this is the first sample of this species that we collected for this project.

To obtain clean sequences from the remaining sponges, the COI gene that was amplified by polymerase chain reaction (PCR) has been cloned into a vector for sequencing. The vector is then transformed into bacteria to grow up more of the vector with the COI insert, followed by isolation of the vector and insert back out of the bacteria. This approach helps to only get a single product for the COI gene and the gene can readily be sequenced within the vector using vector specific primers (e.g. M13 primers). This approach has worked well previously for obtaining clean sequences from complex samples like those of freshwater sponges. The sequencing from the remaining cloned samples is ongoing.

The previously extracted nuclear DNA from each of the sponge samples collected in 2017 has been used to amplify the 18s rRNA gene. The 18s rRNA gene has been amplified from the nuclear DNA that was isolated from all of the sponge samples collected in 2017. To increase the possibility of obtaining clean sequences for the 18s rRNA gene, all of the amplified 18s rRNA gene has been cloned into sequencing vectors. The sequencing of the vectors for each sample will likely be completed in the fall. Once clean sequences for the COI and 18s rRNA are obtained, both genes will be used for robust species identification.

Activity 1 Status as of February 1, 2019:

The Schroeder lab, with the help of four undergraduate students, have completed the spicule preparation and analysis for all 41 sponges collected in Minnesota. The spicules are used for identifying sponge species based on morphology. Although using spicule morphology to identify sponge species is not as robust as genetic identification, it is still an important component in properly identifying sponge species. Megasclere and microsclere spicules from each sponge have been photographed. Measurements (length and width) of the prepared spicules are now being completed using the measurement software associated with our Zeiss microscope. We have also completed the preparation and analysis of gemmuloscleres for half of the sponges collected that had gemmules associated with them. For half of the sponges collected in the spring, we will be completing the gemmulosclere preparation and analysis.

The Schroeder lab, with the help of undergraduate students, has also completed all of the DNA sequencing for the 41 sponge samples that have been collected. The sequences obtained for each sponge has been for the 18s and 28s rRNA gene and for the cytochrome oxidase I mitochondrial gene. These sequences are being used for the genetic identification of the collected sponges. There are still a few sequences from sponges that still need to be performed again from a cloned vector because some are coming back from the green algae that live in symbiosis with the sponge, rather than the sponge itself. Sequences obtained from algae show a second sequence associated with it which is likely that of the sponge. We will be continuing to work to get only the sponge sequence this spring as well.

BLAST analysis of the DNA sequences returned from sponges showed we have found a diverse group of sponges. Our preliminary screening of the BLAST results have identified seven of the collected sponge species as *Spongilla lacustris.* We have initially identified six samples as *Ephydatia muelleri* and one sponge sample as *Ephydatia fluviatilis*. We had six DNA sequence results from BLAST that returned as Spongillidae species, but couldn't provide any information at the genus or species level. Spongillidae is the family level scientific classification for freshwater sponges and these DNA sequences will be prioritized for the evolutionary analysis to determine a finer level of biological classification. We have preliminarily identified two sponges as *Eunapius subterraneus* and another as a Eunapius species, with the BLAST results only identifying the genus, but couldn't determine the exact type of species from the sequence information. This DNA sequence will also be prioritized to determine the specific Eunapius species. Lastly, we have preliminarily identified a collected sponge as a Corvomeyenia species. Similarly to the Eunapius sponge, the BLAST results could only provide resolution to the genus level, so this sequence will also be prioritized for evolutionary analysis to determine the species of the sponge.

To be confident in the identification of the species of sponge, and to determine if the sponge may be of a new species, we have to complete a full evolutionary analysis of the DNA sequences obtained from the sponges. We will be starting this analysis this spring. The DNA sequences we obtained will be statistically compared with sponge sequences that are publically available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). This analysis of individual nucleotide changes in the DNA sequence will allow us to determine if the collected sponges are of different species.

Activity 1 Status as of August 1, 2019:

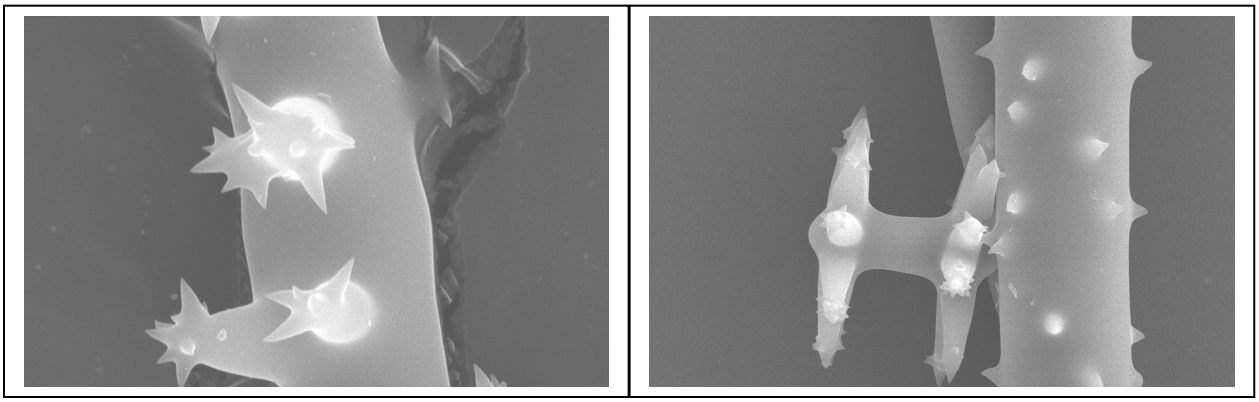
Karl Anderson, with the help of students have completed searching for sponges in rivers in all 10 major watersheds of Minnesota. Lakes in most of the major watersheds have also been sampled. An additional 42 sponge samples have been collected this summer, bring the total sponges collected from the project to over 80 sponges. The over 80 sponges have been collected from a wide variety of rivers and lakes; however very few have found in southern Minnesota and none found in the Lake Superior watershed. This is despite the fact that we have sampled in these

areas multiple times. We plan to try sampling other rivers and lakes in these watersheds to see if we can find more sponges from these areas.

The Schroeder lab with the help of three undergraduate students have separated the 42 collected sponges during the 2019 summer for morphological, genetic, and chemical analyses. The students have completed the spicule preparations for all 42 samples using nitric acid and bleach to prepare the spicules. The slides have been archived and the spicule analysis and photographs for these collected sponges will start in the fall. The DNA extraction for all the 42 samples has started and is completed for some of the samples. Also, the PCR to amplify genes for species identification has also started, but is not completed for all of the sponges. This will be completed for each sponge and then the amplified samples will all be prepped for DNA sequencing. It is expected that all samples will be extracted, PCR amplified, and sequenced early in the fall semester. The DNA sequences from these samples will provide a larger dataset for which we will complete our evolutionary analyses to determine potentially new species.

Activity 1 Status as of February 1, 2020:

We have collected a total of 104 individual freshwater sponges. The Schroeder lab with the help of three undergraduates completed all of the nitric acid and bleach spicule preparations for the collected sponges. A new undergraduate student is working on finishing all of the images of the spicules, as well as completing the measurements for the spicules from each sponge. The spicule measurements are especially important for the freshwater sponges that appear to potentially be a new species. We are also using the prepared spicules for scanning electron microscopy (SEM). SEM provides high resolution images that will further help us to describe the morphology of the sponges and to identify species (see some of the images below).



We have also completed the DNA extractions for all of the collected sponges. All of the sponges have been sequenced at least once for cytochrome oxidase and the 18s rRNA gene. We are cleaning up DNA from samples that didn't provide a clean sequence, but most of the samples we have a good DNA sequence for. It is expected we will be able to have clean DNA sequence for all of the collected sponges prior to the completion of the project. From the DNA sequences, those for which we haven't been able to resolve to the species level we are continuing to perform a full evolutionary analysis for these samples. This will let us determine if the sponge is truly a new species and where it falls in terms of the phylogenetic relationship to other identified freshwater sponges. Based on our initial analyses, we have the potential to identify four or five new species of freshwater sponges collected during this project.

We have the collected sponges from two rivers and two lakes in each of Minnesota's ten watersheds. An undergraduate student majoring in Natural Resources is helping us to perform the GIS mapping of the forty locations where we collected freshwater sponges in the state. It is expected that this full mapping will be completed by the completion of the project. We are also using the GIS mapping with our other collected physiochemical data to try to predict the types of waters where sponges are likely to be found.

Activity 1 Status as of August 1, 2020:

As mentioned in the February report, much of the outcomes for this activity have been completed. The SEM work came to a complete stop when the lab shut down and will hopefully be able to be completed before the project end. As the lab reopens, we are also working to finish the last of the imaging of the spicule morphology for all of the sponges collected during the summer of 2019. The mapping was completed, but we are having additional maps made to show additional data.

Due to social distancing requirements in the laboratory, we have also used the summer of 2020 to perform more sponge sampling in Minnesota. We have sampled additional rivers in each of the ten water basins throughout Minnesota. In fact, we have sampled almost every major river that was present on the water basin map that was used as the visual in the initial proposal. This additional sampling provides a more thorough indication of where sponges are found in Minnesota. We especially focused sampling efforts in southern Minnesota, because our previous sampling provided very few sponge samples in this region. Interestingly, there were still no freshwater sponges found in rivers in southwest Minnesota, south of the Minnesota River. A few sponges were found in south central and southeast Minnesota, but as we found previously, the overall abundance of sponges in southern Minnesota is much less than central and northern Minnesota. We anticipate the water analyses will

help provide some indication of why there is a difference in abundance of freshwater sponges in these different regions of Minnesota. We have collected almost 40 more sponge samples this summer, with plans to sample a few more locations as the lab fully reopens. We are hoping that all the information from this summer's sampling can also be included in the final report. However, we are prioritizing completing all of the spicule imaging from last year's sponge collections. We are planning to have additional maps made with the additional sampling locations from this summer included, provided we can complete the rest of the biological and chemical analyses from this year. Otherwise, we will include as much information from this year's sampling in the final report as an additional appendix.

The samples collected this summer have been processed for spicule morphology and the DNA extracted. As mentioned, priority is being given to completing the imaging of the spicules from last year's collected sponges. If time allows, the processed spicules from sponges collected this year will be imaged. Also, if time allows, the DNA extracted will be used for species identification from this year's collections.

Activity 1 Status as of February 1, 2021:

The majority of the outcomes for activity 1 have been completed. We did perform additional scuba diving for sponges in different lakes throughout Minnesota. We were able to have some success collecting sponges in the lakes sampled by scuba diving.

We are working to finish up the last of the spicule imaging that we need to complete. We still need to finish imaging the spicules that were processed during the summer of 2020. The DNA that was extracted from the sponges collected in the summer 2020 has been used for PCR for cytochrome oxidase I and 18s; however these the PCR products from these sponges need to be sequenced. We are also using the last few months of the project to see if we need to re-sequence any of the sponge samples that were previously sequenced. This is to make sure that we have good clean sequences for following up with the evolutionary analyses.

While we are working on finishing up the last of the imaging and molecular work for the sponges more recently collected, we are also now going back to complete the evolutionary analysis work that we had started previously for sponges that we identified as potentially being new species. This work will continue through the spring, but we are hoping to be able to present in our final report the finding of a couple new species of sponges from this work.

As mentioned in the project status, the mapping of locations has also been completed with locations where sponges were collected prior to this year's sampling. A student is working to also include the locations for the sponge collections from the summer of 2020. It is important to note from this research project, we were able to collect over 160 sponge samples from over 80 locations statewide. This makes this research project the most exhaustive of any sponge sampling that has ever occurred in Minnesota, and yet it turns out there is still much we don't know about these animals.

Final Report Summary:

At the end of the project period, we have completed the most exhaustive sampling of freshwater sponges to date. We sampled 105 locations throughout Minnesota during the project period. We collected sponges from 80 locations, consisting of both lakes and rivers (Figure 1). This resulted in a 76% success rate of finding sponges in Minnesota lakes and rivers that were sampled during the project period. We were able to collect sponges in significantly more than the two rivers we proposed within each watershed. We were able to sample at least the two lakes from each watershed as we proposed. We had greater success finding sponges in rivers than in lakes, but that is likely due to simply sampling rivers more often and that lakes can be significantly deeper. For this reason, we also performed sampling by diving in lakes. We had mixed results associated with diving for sponges, but it seems that more targeted sampling of lakes in the future can produce more sponge sampling. Our future plans as a part of the next grant will focus heavily on sampling lakes and implementing a more targeted lake sampling.

We were able to collect 169 freshwater sponges total across the ten watersheds (Table 1). This is significantly more than we proposed to collect, but previous studies had limited sampling and only found a few sponges. Therefore, we didn't know what to expect and so we proposed a collection number that we felt confident that we could achieve. The greatest number of sponges were collected from rivers within the Upper Mississippi River watershed, followed by the Red River of the North watershed. The reason for the Upper Mississippi watershed having the most sponges collected is likely due to how large the watershed is. The Red River of the North Watershed likely had as many sponges collected as it did, simply because of proximity to the University of Minnesota Crookston and so we could sample these areas more readily. Although the basins in south central and southwestern Minnesota were generally smaller, even when we sampled rivers in these areas of Minnesota over multiple years, we found limited sponges in these locations. The water chemistry at these locations have yet to explain this difference, but we are continuing to perform analyses to explain this difference. Clearly, the landscape in southern central and southwestern Minnesota is different than that of north central Minnesota, where we found the majority of our sponges. We didn't sample rivers in south and southwestern Minnesota until a little later in the project, so we are finishing analyzing the water data associated with the water collected from these rivers and lakes.

We were able to complete the morphological and molecular identification of the sponges that were collected through 2019, that we said we would finish in outcome 2. However, we did also collecting sponges beyond 2019. We were able to prepare spicule preparations for all of the sponges that we collected. We were not able to get all of the spicules imaged and photographed from the sponges collected in the summer of 2021. All other spicules have been imaged and the megascleres and microscleres (when present) have been measured. The spicules are used in combination with the sequence analysis for determining sponge species. All sponge samples have had the DNA extracted from them. The sequencing analysis was not finished for all of the samples collected in 2021. However, this will be work in continuing and will be completed in the fall of 2021. The other sponges whose DNA has been extracted have also been sequenced for the barcoding gene, Cytochrome Oxidase I (COI). Using COI, we identified the majority of sponges that were collected were those previously identified in Minnesota, including *Ephydatia mulleri, Ephydatia fluviatilis, Eunapius fragilis, Spongilla lacustris*. We also found *Trochospongilla pennsylvanica*, which is less common in Minnesota, but has been reported less frequently than the other sponges previously listed. We also found two sponges that were only able to be identified to the genus level, which was *Corvomeyenia*, but not the species level. The genus *Corvomeyenia* has not been an identified genus of sponge in Minnesota. We have completed the evolutionary analysis and finishing scanning electron microscopy (SEM)

images to confirm that the sponge found is a new species. We also found six sponges that we could only identify to the family level, spongilladae. For these samples, we need to obtain a DNA sequence for the COI gene. We are continuing to work on this, as it is likely that these will be new sponge species as well. We will also be obtaining SEM images of the spicules from these sponges to further support the hypothesis that these are new species.

Overall, we were able to complete pretty much all of the outcomes associated with activity 1. We were able to sample at least two, and in reality significantly more, bodies of water in each of the watersheds. We also were able to identify the majority of the sponges we collected prior to the summer of 2021. Also, we have almost most definitely found one new species of sponge in Minnesota from the Ottertail River, and likely found an additional 3 or 4 new species. Therefore, because of this project, it will contribute additional species to only the 32 species of freshwater sponges that have been identified in North America.

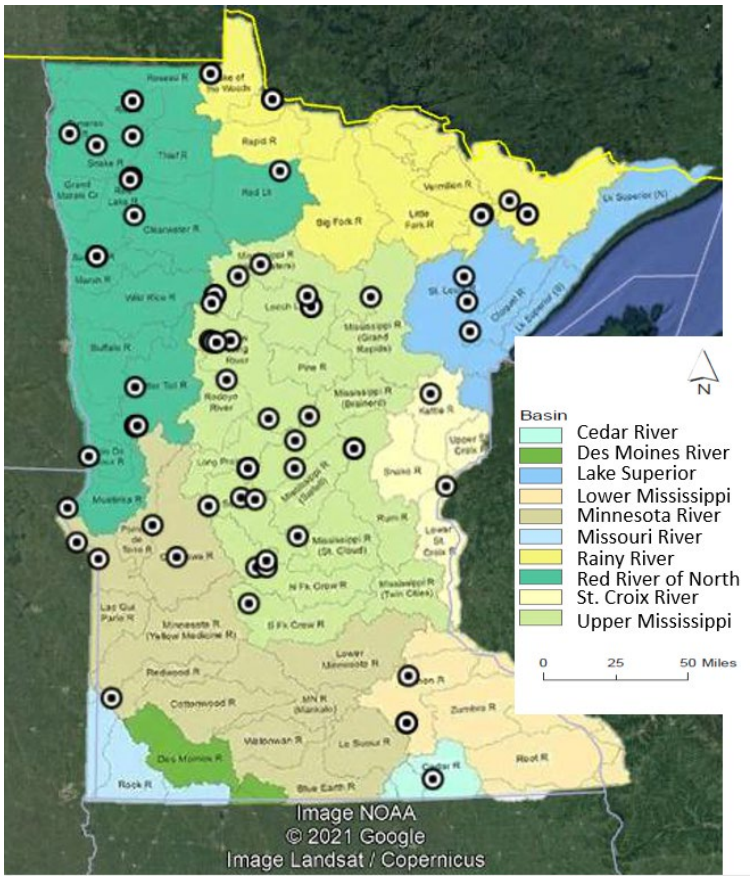


Figure 1. Map of the distribution of freshwater sponges collected across the ten water basins in Minnesota. The black dots indicate each location.

Table 1 : Breakdown of the total number of sponges collected in either lakes or rivers within each of the ten water basins in Minnesota.

Watershed	Location Type	Sponges Collected
Cedar River	Lake	0
	River	1
Des Moines River	Lake	1
	River	0
Lake Superior	Lake	1
	River	18
Lower Mississippi	Lake	0
	River	4
Minnesota River	Lake	2
	River	4
Missouri River	Lake	1
	River	0
Rainy River	Lake	3
	River	9
Red River of the North	Lake	0
	River	29
St. Croix River	Lake	0
	River	2
Upper Mississippi River	Lake	3
	River	91
Total Sponges Collect		**169**

ACTIVITY 2: Detection of contaminants present in collected freshwater sponges

Description:

Collected sponges will be freeze-dried and organic material will be extracted with suitable solvents such as hexane, dichloromethane and methanol. Chemical standards will be used for the identification and quantification of contaminants. The presence of PAHs, PCBs, and other emerging contaminants such as pharmaceuticals, pesticides, and estrogenic compounds in the sponge will be determined using EPA protocols when available. For example, the Environmental Protection Agency (EPA) has a number of protocols for qualitative and quantitative determination of PAHs. We will adapt EPA method 550.1 which is used to determine PAHs in drinking water using HPLC and EPA method 525.2 (Revision 2.0) using GC-MS. In preliminary studies, 14 of the 16 compound mixture of PAHs bought from Sigma-Aldrich were clearly separated using a reverse phase Zorbax C_{18} column (250 X 4.6 mm) with an acetonitrile-water gradient at a flow rate of 2.0 mL/min. The compounds were detected at 254 nm using a diode-array detector. A similar analysis will be performed for inorganic contaminants.

Summary Budget Information for Activity 2:

ENRTF Budget:	**$92,500**
Amount Spent:	**$ 84,805**
Balance:	**$ 7,695**

Outcome	Completion Date
1. *Extraction of contaminants from collected freshwater sponges*	January 2019
2. *Identify and quantify contaminants present in sponge extracts using established analytical methods (e.g. EPA protocols)*	June 2020

Activity 2 Status as of February 1, 2018:

Drs. Mukku and Dudley, with the assistance of 3 undergraduate students, have started extracting the contents of the freeze-dried freshwater sponges collected to date. The contents of one sponge sample has been extracted using three common solvents: hexane, dichloromethane, and methanol. This process is currently being used to extract the contents of the remaining sponge samples. Another undergraduate student is currently developing procedures for separating the chemical components of the extracts and preparing them for further chemical analysis (e.g., HPLC).

Activity 2 Status as of August 1, 2018:

The 2.5L Labconco freeze-dryer that was asked for activity 2 was purchased with the requested $10,000. The freeze-dryer has been used to prepare all of the sponge samples for downstream chemical extractions.

Drs. Mukku and Dudley, with the help of three undergraduate students during the spring semester and one undergraduate full-time during the summer, have performed extractions for all of the sponges collected in 2017. Extracts have been prepared using the solvents: hexane, dichloromethane (DCM) and methanol for each sponge.

NMR spectra of hexane, DCM and methanol extracts from three sponges were recorded in d6-DMSO (deuterated dimethyl sulfoxide). The spectra were analyzed using MNova for deciphering common features. Preliminary analysis of the stacked NMR suggests that the three hexane extracts contain steroids. Previous researchers reported isolating a number of steroids from freshwater sponges. The stacked NMR spectra and individual NMR spectra do not reveal the presence of PAHs and PCBs. However, NMR is not a very sensitive technique and needs complementary data from mass spectrometry.

Standards have been purchased for contaminants we said we would examine within activity 2. The standard samples are categorized into five categories namely 1) PAHs (2) PCBs (3) Pharmaceuticals (4) Pesticides and (5) Estrogenic and others. A total of 13 standards including PAH and PCB mixtures were bought from Sigma-Aldrich. Sertraline Hydrochloride, Sulfamethoxazole, Metformin hydrochloride and Iopamidol comprise the pharmaceutical group. Glyphosate, Atrazine form the pesticide group. DEET, bisphenol A, 5-methyl 1-H benzotriazole and pentadecafluorooctanoic acid (PFOA) comprise the estrogenic and other group.
The standard samples will be run on a LC-MS (liquid Chromatography coupled to a mass spectrometer) at different concentrations (10 ppm to 1 ppb) to ascertain their individual retention times under a given set of conditions (type of column, solvents, flow rate and detection parameters). Once the retention times of standard compounds are determined, the sponge extracts will be analyzed under identical conditions. This will allow our group to determine if sponges have sequestered these chemicals from the waters.

We are also currently and in the future working on optimizing our techniques for examining organics and inorganics from the water samples collected.

Activity 2 Status as of February 1, 2019:

All of the collected sponges from the summer of 2018 have been freeze-dried and are ready for downstream chemical extractions. Undergraduate students helping with the project are working on preparing extracts for these using the solvents: hexane, dichloromethane (DCM) and methanol for each sponge.

The dichloromethane and methanol extracts of four sponges (sample numbers 1002 (Rum River near Mille Lacs), 1003 (Ottertail River near Fergus Falls), 1010 (Mississippi River near Grand Rapids) and 1011(Mississippi River near Grand Rapids)) collected in the summer of 2017 were analyzed by GCMS. Three (1003, 1010 and 1011) of the four sponges were green which suggested algal presence in the sponges. GCMS data showed that these sponges contain neophytadiene and related compounds that are normally produced by algae rather than sponges.

The mass spectra of sponge metabolites in each chromatogram were compared to those included in the NIST Atomic Spectra databases. Compounds with higher than 85% similarity were identified (See attached Excel spreadsheet for specific compounds identified in each sponge). Long chain alcohols, aldehydes, amides, cholesterol and benzenepropanoic acid were common to many of the sponge extracts. Of particular note, is the occurrence of hexadecanamide, palmitoleamide and oleamide in sponges 1003, 1010 and 1011.

It is difficult, at this point, to determine the origin of these metabolites (sponge or algal). Oleamide was reported to be used in antifouling paints, is known to be leached from certain plastics and was also reported to be involved in thermoregulation, inducing sleep.

Since it is present in many extracts an authentic sample was bought and analyzed by GCMS under identical conditions. The retention time of the authentic oleamide sample matches with the sponge metabolite (identified as oleamide by similarity search). A sample of hexadecanamide was also bought for comparison but we have not analyzed its retention time yet.

Literature search of sponge metabolites are sparse with reports of long chain amides. However, there is one report we are aware of that identified octadecanamide from Brazilian and Spanish marine sponges.

Activity 2 Status as of August 1, 2019:
Thirteen freshwater sponges collected in the 2018 season were freeze-dried and extracted sequentially with dichloromethane and methanol. The extracts are ready for analysis by GC-MS and LC-MS. The rest of the 2018 freeze-dried sponge samples are being extracted, followed by the 2019 freeze-dried samples.

Water samples have been collected from each of the locations where sponges were found. The water samples were separated to analyze organic compounds, inorganic compounds, and metals. For analyzing organic compounds, the water sample is being processed through solid phase extraction (SPE). The samples prepped through SPE will be analyzed by GC-MS and LC-MS. For the metals, the water sample is being acidified and being prepped for analysis using the ICP-MS. The water samples prepped for inorganic analysis will be analyzed using the ion chromatograph. These different analyses are just beginning, and will continue through the rest of the year.

Activity 2 Status as of February 1, 2020:
All of the 104 freshwater sponge samples have been freeze-dried to prepare them for chemical extractions. The majority of the sponge samples have been extracted using both dichloromethane and methanol. The remainder of the sponge samples will be extracted this spring. All the extracts are being analyzed using GC- and LC-MS.

All of the physiochemical and total organic carbon (TOC) analyses have been completed for the water samples that were collected for where freshwater sponges were found. This data will be incorporated with the mapping of freshwater sponge locations that is being performed in activity 1.

The water collected water samples are continuing to be processed through solid phase extraction (SPE) to identify chemicals that are present in the water, and also within the sponges where the water was collected. The extracts from water prepped through SPE are being stored and will be analyzed using GC- and LC-MS also.

Activity 2 Status as of August 1, 2020:

As mentioned, the preparation of chemical extracts from collected freshwater sponges came to a halt when the lab closed in March. As the lab reopens, we have prioritized completing the sponge chemical extractions.

The chemical extracts from water samples that were being prepared through solid phase extraction (SPE) also came to a halt. The chemical extracts from water will resume as the lab fully opens. We suspect that we will be able to complete all of the chemical extracts from water and sponges, and perform the chemical analyses prior to the completion of the project.

Water samples that were collected from each of the sampling locations have sent out to the water analysis lab at the University of Minnesota for element analysis using ICP-OES. This will provide us with the parts per million concentrations of elements in the water that may influence the presence or absence of sponges in Minnesota's rivers and lakes. This analysis will be included with a larger dataset containing additional physicochemical data from each sampling location.

The sponge samples collected during the summer of 2020 will be freeze-dried and stored for chemical extractions. However, these samples will not be prioritized for processing and can be stored for future extractions. Also, the water samples collected during the summer 2020 sampling have been prepped for long term storage to allow for future analyses.

Activity 2 Status as of February 1, 2021:
The collection of chemical extracts from the sponges has been a priority since the August report and will continue to be a priority through the spring semester. We are hoping to prepare chemical extracts for analysis for all of the sponges that we have collected through the summer 2020. We have put additional students on this work to hopefully get through all of the samples collected; however, it is expected that we will have extracts prepared and analyzed for all sponges collected through the summer of 2019.

We have received the element data using ICP-OES from the University of Minnesota for all water samples collected. Those data are being analyzed and along with other physiochemical data obtained from collection locations. We are also working to finish up the other water analyses including total organic carbon and ion chromatography (ions not examined with ICP) for the water samples.

All sponge and water samples have been prepped for long term storage in case the samples are not able to be analyzed prior to the completion of the project in June. It is still expected that these samples will be analyzed and be a part of a larger dataset for a future manuscript.

Final Report Summary:

All of the freshwater sponges that we collected have been freeze-dried and archived so that we have them for any further analyses needed to complete. We analyzed the physiochemical data of the water from where sponges were collected and where we sampled, but sponges were not found. We also completed measuring the total organic carbon in the water from the locations were sponges were collected and not collected. For these analysis, there does not appear to be any association with the physiochemical properties of the water nor the amount of total organic carbon to explain the presence or absence of freshwater sponges in rivers in Minnesota. We are finishing analyzing the full ICP data for 27 different elements to determine if any of the elements or a set of elements are associated with the presence or absence of freshwater sponges in Minnesota's waters.

Chemical extracts have been prepared from the freshwater sponges that were collected through the summer of 2019. We still have extracts that need to be prepared for sponges collected last summer, but are continuing to work through these. The extracts that were prepared have been analyzed using GC-MS. We didn't identify the presence PCBs nor PAHs in the sponges sampled, thus far. This suggests that sponges are not accumulating these contaminants and are likely not having a biomagnification effect of the contaminants through the food chain.

Our chemical analysis of freshwater sponges identified interesting long chain fatty acids. These fatty acids may be directly from the sponges or may be from the bacteria associated with the sponges. We are working to determine if the fatty acids are specifically from the sponges. We are also looking at other compounds that have been identified in freshwater sponges and the potential of these compounds to be bioactive and be used for human purposes.

ACTIVITY 3: Training undergraduate students in interdisciplinary research and dissemination of research findings to the appropriate stakeholders

Description:

During year 1, 4-6 students will learn how to collect and identify sponge specimens using accepted techniques (Activity 1). During year 2, 4-6 students will learn how to perform chemical analyses on these specimens using appropriate laboratory techniques and instrumentation (Activity 2). By the end of year 3, 6-8 students and 2-3 faculty will have disseminated much of their findings to interested state agencies (e.g., MPCA) and at professional conferences by way of poster and/or oral presentations or white papers. Based on the results of this project, certain aspects of this work will be incorporated into laboratory courses from freshman-level in biology, chemistry. Students engaged in this research will be well prepared for required internships and future employment.

Summary Budget Information for Activity 3:

ENRTF Budget:	**$50,000**
Amount Spent:	**$50,000**
Balance:	**$ 0**

Outcome	Completion Date
1. *10-12 Students will be trained in proper field collection and laboratory procedures*	June 2019
2. *6-8 Students and 2-3 faculty will disseminate research findings to potentially interested state (MPCA, MN DNR) agencies and scientific conferences*	June 2020
3. *Integration of sponge research into undergraduate curricula (e.g., biology, chemistry) classes*	June 2020

Activity 3 Status as of February 1, 2018:

The Schroeder lab has facilitated the work of three undergraduate students. These students are working with Dr. Schroeder to learn proper laboratory procedures for the morphological and molecular identification of sponges. Drs. Dudley and Mukku have also included three undergraduate students in their research since the start of the project.

Aspects of the sponge research have already started to be incorporated into the Biology undergraduate curriculum. Sponge collections, DNA extractions, and spicule analysis were incorporated into the Biology's Nature of Life course in fall 2017. Students were exposed to aspects of aquatic ecology in the field, as well as sample transport, storage, and preliminary microscopy work. DNA sequence analysis from freshwater sponges is also being incorporated into the Biology program's Molecular Biology Techniques course in the spring 2018.

As stated earlier, an unexpected outcome of this project has been the interest in this project by the public. In order to better inform the public, Drs. Schroeder, Mukku, and Dudley are working with faculty from the Communication program at UMC to develop effective ways to communicate our work with the public. One faculty member, Dr. Megan Bell, is incorporating development of materials (e.g., pamphlets) into her publication design and management course for spring 2018.

Activity 3 Status as of August 1, 2018:
The Schroeder lab has had a total of four undergraduate students who have worked on the project since the last report in February. Each of the students have been learning proper laboratory procedures for the morphological and molecular identification of sponges. The students have been leading the work for the amplification and cloning of the 18s rRNA gene for sequencing. A little over a year into the project, the Schroeder lab has had the opportunity to have six different students be involved in the project, with each learning about the morphological and molecular analysis of freshwater sponges.

We have continued to incorporate aspects of the freshwater sponge project into the Biology undergraduate curriculum. In the spring, the process of DNA extractions and PCR using COI primers was incorporated into the Molecular Biology Techniques class. Students also performed DNA sequencing of the PCR products they amplified using the COI primers.

Steven Gonazalez, an undergraduate student involved in the freshwater sponge project, had a story highlighting his participation in the freshwater sponge project present on the University of Minnesota's News and Events main page. An additional story about Steven's role in the project was highlighted in a story in the University of Minnesota's Foundation publication, Legacy.

Activity 3 Status as of February 1, 2019:
The Schroeder lab has four undergraduate students who are working on the freshwater sponge project. Three students are continuing to work on the project from over the summer (Trevor Long, Michaela Lano, and Kaitlin Sikkink) and one additional student (Riley Thompson) is working on the project. The students are continuing to develop skills in molecular biology and will begin to learn evolutionary analysis for the possibility of identifying new sponge species.

Trevor, Michaela, and Kaitlin had an article highlighting their involvement in the freshwater sponge project in the Fall/Winter 2018 edition of the University of Minnesota Crookston's Torch Magazine (https://issuu.com/umcrookston/docs/torch_fall_2018). Trevor, Michaela, and Kaitlin have also submitted and received an accepted abstract to present their research from the sponge project at the National Conference on Undergraduate Research (NCUR) in April.

We have continued to work to incorporate freshwater sponge research into the Biology and Chemistry curriculum at UMC. In the fall of 2018, aspects of the freshwater sponge project were included in the Biology program's Nature of Life course. Students were able to perform sponge collections, water collections, DNA extraction and PCR using material from their collections. Drs. Dudley and Schroeder are also incorporating aspects of the freshwater sponge project into a Biology special topics they are teaching during the spring 2019 semester. The nine students in the class are learning aspects of the chemical analyses associated with the sponge project. The students are also learning more about sponge biology and their lifecycle and how the water chemistry can affect their lifecycle.

Four undergraduate students (Kaitlin Sikkink, Darin Viken, Morgan Kresl, and Lindsey Weber), as a part of their Chemistry 3994 course, have also been preparing and analyzing water samples from where the sponges were collected as to determine if there are specific water quality parameters that may help us predict where sponges would be found in Minnesota waters. UMC has recently received a grant that allowed for the purchase of two chemical instruments to allow us to perform analyses on the collected water samples. Total organic carbon (TOC) content has been analyzed for 25 water samples that were first filtered using a 0.40 micron filter to remove undissolved particles. Thus, dissolved organic carbon (DOC) was measured for each sample and the values ranged from 5 parts per million (ppm) to over 200 ppm. While many of the samples contained typical DOC levels for lakes and streams (5-10 ppm), a number of samples contained well over 20 ppm of DOC. For those samples that had > 20 ppm DOC we used a 0.22 micron filter to try to further remove undissolved particulate matter. While this did reduce the DOC levels significantly for many of the samples with the highest DOC, the detected DOC levels were still high (> 40 ppm). Preliminary analyses of the inorganic content of these waters samples has also been initiated. pH and conductivity measurements have been recorded for the filtered water samples that had low TOC readings (< 15 ppm). The pH readings show these water samples as being somewhat basic with a pH between 8 and 9. The conductivity of these samples ranged from 130 to 430 microSiemens per cm. These samples were chosen as examples of "pristine water" based on their TOC content and the pH and conductivity values reported corroborate this assumption. Future analyses of inorganic content will focus on the specific content of these samples (i.e., identities of cations and anions). This will be done using a newly acquired ion chromatography (IC) instrument that can both identify and quantify ions in water samples.

Activity 3 Status as of August 1, 2019:

Three undergraduate students (Kaitlin Sikkink, Michaela Lano, and Trevor Long) presented their work on this project at the National Council of Undergraduate Research (NCUR) meeting in Kennesaw, Georgia from April 10-13, 2019. They presented results of their work on both the biology and chemistry aspects of this project through a poster presentation.

Michaela Lano, an undergraduate working on the project, presented a poster at the UMC Research Fair on April 18, 2019. Michaela presented the biology and chemistry results from the freshwater sponge project to students and faculty at the University of Minnesota Crookston.

Three new undergraduate students (Boyce Harr, Riley Thompson, and Hee-In Moon) have started working on this project. They have been involved with locating and collecting sponges from Eastern and Southern Minnesota. Riley has been involved in the sponge morphology and genetic analysis work. Boyce and Hee-In have been involved in the processing of sponges for chemical analysis by freeze-drying them and extracting the contents of the sponges using various solvents. Boyce Harr has also started processing water samples collected

at the locations where sponges are found. These samples will be analyzed for various organic compounds, metals, and other inorganic species (e.g., nitrates and sulfates) to provide a snapshot of the environment these sponges are exposed to.

Activity 3 Status as of February 1, 2020:

The Schroeder lab had two undergraduates who previously worked on the project graduate. A new undergraduate student has joined the lab to help complete the imaging and measurements of the spicules. Drs. Mukku and Dudley also had a student graduate and a new undergraduate student is helping complete the chemical analysis. We have also have a student in Natural Resources helping with the mapping associated with the project. This puts the number of undergraduate students that have been involved in the project at around the twelve students that we were hoping to train on this project.

Trevor Long, an undergraduate working on the project, provided an oral presentation about his sponge research to the University of Minnesota Campus during the fall research day (December 5, 2019).

In January, three students (Michaela Lano, Trevor Long, and Riley Thompson) presented their sponge research at the 37th Annual Red River Basin Land & Water International Summit Conference. The students were invited to present their sponge research at this conference.

Activity 3 Status as of August 1, 2020:

The species identification for the collected freshwater sponges was going to be incorporated into the Molecular Techniques during the spring 2020 semester. However, classes went online in March and so students were unable to participate in the species identification portion of the project.

Due to COVID-19, we were unable to bring new students into the lab to work on the project this summer. Two undergraduate students who have previously worked on the project have continued to work when the lab reopened this summer. The new students will be brought into the lab this fall when the lab fully reopens, but under the restricted guidelines to allow social distancing. Thus, we will have less new undergraduate students working on the project than originally anticipated.

Activity 3 Status as of February 1, 2021:

As the lab has been able to fully reopen while following COVID restrictions, we have been able to have three additional undergraduates work on the project. One new student is working on the microscopy work (finishing up activity 1) and the other two students are focusing on the chemistry and chemical analyses (activity 2). Each of these students have interests in these areas of research, so participating in this research is providing them the opportunity to develop additional skills in this area.

We have been communicating and working with researchers with the 1854 Treaty Authority in North Eastern Minnesota about where we have collected sponges and where they have seen them as well. The researchers have expressed interest in sponges in Minnesota and their role in the ecosystem, so we plan to continue working with this group in the future.

Final Report Summary:

One of the things we are most proud of associated with this project is the number of students we were able to involve in this project. Between the biology and chemistry areas associated with this project we were able to train eighteen students in research because of this project. The experiences that students had because of this project has also helped students achieve their goals after graduating. Two students who were involved in research have gone on to purse Doctor of Philosophy in Biology related fields. Two students are pursing Masters Degrees in fields of Biology. One student is also teaching science to high school students in the Twin Cities area. We also had students who are pursuing Masters in Nursing, Physician Assistant School, Medical School, and Pharmacy School. We were able to help these students by writing letters of recommendation for them because we were able to get to know these students well because of this research project. This project has helped to prepare the future doctors, scientists, nurses, and teachers for Minnesota, as well as other states.

This project also allowed the students to be able to present the findings of this project to various interested parties. Three students were able to present their results at the National Conference of Undergraduate Research. Three additional students presented at regional meetings to present to stakeholders that were interested in water and water quality in the Red River valley and other areas in Minnesota. The faculty involved in the project also presented the results to various stakeholders. Much of the presentation was to the general public, as well as to people interested in water and water quality in Minnesota. This included presenting to Master Naturalists associated with the University of Minnesota Extension. Further explanation of the dissemination of the research and results of this project are provided in section V. Dissemination below.

Lastly, we were able to successfully integrate aspects of this project into the classroom curriculum. The Molecular Biology Techniques class at the University of Minnesota Crookston (UMC) has now added the barcoding, sequencing and sequencing analysis of freshwater sponges completely into the curriculum. Also, the Analytical Chemistry course at UMC now incorporates techniques and data production that we used for this project and will continue to use in the future. Therefore, because of this project, we are able to train significantly more students into the future in Biology and Chemistry techniques that we never could have done as well or as successfully if we hadn't received this funding from LCCMR.

V. DISSEMINATION:

Description: The results of the work outlined in this proposal will be disseminated in a number of formats and venues. Since undergraduate students will be heavily involved in this work, they will be presenting their results via poster and oral presentations at any number of local, regional, and national conferences and symposia. UMC hosts an undergraduate research day each year in which students discuss the research projects they work on to their peers and other faculty. Recently, UMC students have presented their research findings at both regional and national meetings (e.g., American Chemical Society, National Council of Undergraduate Research). Similarly, UMC faculty have been presenting at many of these and other conferences (e.g., Society of Environmental Toxicology and Chemistry). We plan to continue our attendance at these conferences in order to disseminate our findings for this project. One new set of venues we anticipate disseminating our results to various state agencies (e.g., DNR, MPCA) that may be interested in our work.

In addition to the traditional dissemination of results, we also anticipate informing the public of our work on this project through various media outlets. The Minnesota Conservation Volunteer has already expressed interest in covering our preliminary work on this project. UMC Public Relations often does stories on student and faculty research, and those stories appear in the local and hometown newspapers of the students involved. As a land-grant institution, UMC is obligated to engage in public outreach and education; thus, we expect that this project will be utilized by our institution to achieve its land-grant mission.

While the scientific merit of this project has been thoroughly outlined, the educational impact of this work is just as important to UMC and its students. Having students engaged in meaningful research is known to help in student retention at both the academic institution and in STEM fields. Thus, we plan on incorporating this project into a number of our biology and chemistry courses to give as many of our students as possible the opportunity to engage in original research. We have already started this process by incorporating some of the methods outlined in this project into our first-year experience (Nature of Life course) designed to introduce students to research-based methods in the biological sciences. During this experience, students visit the University of Minnesota's Biological station for a weekend field-trip. This trip is composed of laboratory and field experiences where students work closely with UMC faculty/staff. This academic year, we chose to involve the students in some basic analyses of sponges that were collected in a region near the park. We intend to expand on the work these students performed in the Nature of Life course in a number of other courses: Zoology, Cell Biology, Genetics, Organic Chemistry, and Analytical Chemistry. The goal of this incorporation is to expose all of our students, who are majoring in biological sciences, to original research. It is hoped that the outcome of this process results in students becoming more engaged and involved in individual research projects.

Status as of February 1, 2018:
We have been working to disseminate the work of this project through multiple sources. The Department of Natural Resources' Minnesota Conservation Volunteer Magazine published an article about the research project in July 2017. The article provided a significant means for Minnesota citizens to learn about freshwater sponges and our project. Our contact information was also provided in the article so citizens could reach out to us. The public response to the article was extremely positive. We received significant comments and information about freshwater sponges in Minnesota from many individuals. The online version of article was shared by over 175 various social media users.

In September, Anthony Schroeder gave a freshwater sponge project presentation at a University of Minnesota of Minnesota Alumni Association event called MN Sparks. The presentation was entitled "Soaking Up the Unknown in Minnesota's Waters." This event was attended by alumni, faculty, and staff from the University of Minnesota, as well as members of the general public.

In October, the University of Minnesota published a system-wide article about the freshwater sponge project, and later published another article which featured a UMC student working on the project. The articles focused on the purpose of the project and the important research opportunities students are receiving because of the project.

In November, Anthony Schroeder was interviewed by Green Sense Radio about the freshwater sponge project. Green Sense Radio is a podcast that focuses on areas associated with protecting and supporting our natural environment. The interview focused on the background of freshwater sponges and how they may function as a bio-indication of clean water.

In December, Karl Anderson was interviewed by Scuba & H20 Adventures Magazine and discussed the overall freshwater sponge project. Scuba & H2O Adventures is a nation-wide publication which broadly covers stories related to watersports. The interview focused both on the background of freshwater sponges and aspects related to the collection of sponges during the research project. The Scuba & H2O Adventures article was distributed to 500+ outlets of the Dive News Network Media Group and shared in several social media platforms.

Status as of August 1, 2018:
We have continued to actively participate in outreach with the general public and state entities and disseminate our research as the project is continuing. Since the last report, our outreach has included communicating and assisting high school students in the state. As far as outreach, we have been contacted and are assisting undergraduate at the University of Queensland, Brisbane, Australia for sponge identification. In Minnesota, we have been contacted by students from Pine River-Backus high school who are interested in learning more about freshwater sponges for a biology project. Gabby Zimprich, a high school student at Sacred Heart High School in East Grand Forks, MN, used freshwater sponges as a part of her science fair project. She presented her project entitled "What Kinds of Bacteria are found on Freshwater Sponges" at the Western Regional Science Fair. Gabby informed people who attended her board about freshwater sponges in the state and what she was able to find for bacteria that are associated with them. Her presentation provided Gabby with a second place finish and a first alternate to the Minnesota State Science Fair. He have also been working with the Minnesota Department of Natural Resources (DNR) to incorporate aspects of the freshwater sponge project with their interests and mission. The DNR has incorporated freshwater sponges into their aquatic geocaching program that they have for Minnesota citizens (see attachment). We have also received an open invitation to tour the DNR's mussel culture facility in Lake City, with the potential for possible collaborations in the future. We have not had the opportunity to take advantage of this invitation as of yet, but hope to in the near future. We have also been approach about presenting aspects of the sponge project at the DNR supervisor's meeting in September. The details of this are still be worked out, but we are hoping it will work with our schedules to present our project and findings to this group.

Status as of February 1, 2019:
A major priority of this project has been to engage with the general Minnesota public and involve them in the project. As mentioned above, the freshwater sponge project was highlighted in the fall/winter edition of the University of Minnesota Crookston's Torch Magazine. The magazine also highlighted citizen scientists, Teresa and Aaron Alto, who became interested in freshwater sponges and contacted the project investigators after reading the article in the DNR's Minnesota Conservation Volunteer. The Alto's have been helpful, providing freshwater sponges reports around the Grand Rapids area. We have also been contacted by six more members of the general public mentioning where it is that they have found freshwater sponges, and have mentioned their overall interest in the project.

The freshwater sponge project was also highlighted in an article published by the Grand Forks Herald (https://www.grandforksherald.com/news/education/4516554-umc-opens-new-collaborative-lab). The article mentions the research being performed by the students as a part of the freshwater sponge project.

We have also continued to be in contact and engaged with Minnesota State Agencies. We have been in contact with Daniel Fettig at the Minnesota Pollution Control Agency. He has provided us with historical information

where the Agency has found sponges during their sampling for fish and macroinvertebrate assemblages. He also included some basic data about the collection sites and also some basic water chemistry data from these locations.

We have also been in contact and working with others throughout the state. For example, we were contacted by Hannah Neusch. Hannah works as a conservation planner for the Martin Soil and Water Conservation District. She believes they found a freshwater sponge on a cattail stalk in one of their CREP wetland sites. We haven't checked CREP wetlands, but it isn't an impossibility that gemmules are present in the wetlands and when the conditions are reasonable, hatch and produce a new adult sponge. As mentioned, southern Minnesota was extremely wet this summer and fall, so it seems likely a freshwater sponge was found in the wetland this past year. She is sending us a sample and we will determine if the sample was a freshwater sponge that she found in the CREP wetland.

We have also been in contact with Joan McKernan and Paula Croonquist from Anoka-Ramsey Community College about using freshwater sponges as a part of their curriculum at Anoka-Ramsey. We met Joan in Grand Rapids in the fall to show her freshwater sponges and provide her sponge samples from the Mississippi River. We will continue to provide any support to help these faculty incorporate sponges into their curriculum so more students at institutions in Minnesota can learn about these animals.

Status as of August 1, 2019:

We have continued to receive questions and reports related to freshwater sponges via email and phone from various individuals across Minnesota. We have also continued to be in contact and engaged with Minnesota State Agencies. The Minnesota Department of Natural Resources continues to express interest in the project.

In March, Karl Anderson shared a project update with DNR officials and members of the Fisheries Citizen Oversight Committee.

In July, Venugopal Mukku, Anthony Schroeder, and Karl Anderson spoke with a number of Watercraft Inspectors in the Bemidji, Lake of the Woods, Rainy River, and Ely areas.

In July, Amy Wilfahrt, Fisheries Biologist with the U.S. Forest Service in Grand Marais, MN, reached out to the research group. She inquired where freshwater sponges could be found as well as what genus and species were most common. We continue to speak with Ms. Wilfahrt and will likely meet with her in-person and search for sponges in the Gunflint Ranger District.

As previously mentioned, 3 undergraduate students presented their work on this project at a national research conference (NCUR, http://www.cur.org/assets/1/7/NCUR_2019_Conference_Program.pdf, page 312).

Anthony Schroeder was invited to North Dakota State (NDSU) to present at the Environmental and Conservation Seminar Series about the freshwater sponge project. Anthony gave the presentation to graduate students and faculty at NDSU on February 21st.

Status as of February 1, 2020:

As previously mentioned, three undergraduate students presented their sponge research at the 37th Annual Red River Basin Land & Water International Summit Conference. They presented their research to members of the Red River Valley community, but also members of Minnesota's Pollution Control Agency and the Minnesota Department of Natural Resources.

We have also been assisting Ashley Moerke at Lake Superior State University who has undergraduate students performing freshwater sponge research. We are helping her provide assistance with freshwater sponge identification and genetic procedures.

We have also been providing assistance to Marc Yergin from the Carnegie Museum of Natural History in Pittsburg, Pennsylvania. He has a project related to freshwater sponges and we have been working with him to discuss how freshwater sponges can function as a useful bio-indicator.

We have also been in contact with Dr. Aeisha Thomas at Crown College in St. Bonaifacius, MN about her using freshwater sponges as a part of her barcoding project at the College. We have discussed the possibility of us sending her sponge samples to allow the students to isolate DNA from them and use it to identify the sponge species.

Status as of August 1, 2020:

Despite the lab being shutdown, we have continued to be in contact with investigators within and outside of Minnesota, who are interested in freshwater sponges. When the lab opened we sent freshwater sponge samples to Dr. Aeisha Thomas at Crown College. She is sending a subset of the samples to Cold Spring Harbor Laboratory as part of a microbe barcoding project. The other samples we sent, she is planning to use as a teaching tool for DNA barcoding in her class this upcoming fall.

He have also been in contact with John Copeland, Tennessee, about sponges present in Minnesota. He is working on a book related to freshwater sponges found in the United States and is inquiring about species we have found from our sampling.

Anthony Schroeder did an interview with the University of Minnesota Extension's Master Naturalist's Podcast about the freshwater sponge project. The interview is scheduled to drop this next month.

Status as of February 1, 2021:

As mentioned in the August report, sponge samples were sent to Dr. Aeisha Thomas at Crown College for her to use as a part of her barcoding project, but also for her to use in her classroom teaching. She sent a summary of the data she was able to collect from the barcoding project and had a good success using the sponges for her project. She also mentioned that the students enjoyed using the sponges from Minnesota for barcoding analyses in her class this fall, and she has asked for us to send additional samples this spring for her to use again in her class this semester. We will be sending her those samples later this month. She has also mentioned that students at Crown College have become interested in sponges and would like to join us for sponge collecting, so we are working to coordinate a trip with them this summer.

We have had an abstract accepted to present our sponge research at the virtual University of Minnesota Extension Gathering Partners Conference this summer.

Lastly, we have had an abstract accepted to allow us to have a research booth at the Driven 2 Discover building at the Minnesota State Fair in 2021. If the fair is held, we will present our research to the general public at the fair and allow them to learn more about sponges in the state.

Final Report Summary:

Throughout the entirety of this project we have worked to disseminate this research through a variety of different mediums. This research has been the focus of six different news articles that were published by different groups ranging from the University of Minnesota, to the Minnesota DNR, to Scuba & H2O Adventures. We also were involved in a number of podcasts that described this research project and its importance. This included podcasts by both public and private entities including University of Minnesota Extension to Green Sense Radio. We also presented this research at both regional and national conferences. In total this research was presented by faculty and students working on this project at six different conferences. There were additional conferences where an abstract was accepted and we were going to present this research; however, the conference was cancelled due to COVID.

Because of our dissemination of this research through a variety of mediums, we also had a large number of people from different institutions reach out to us about learning more about freshwater sponges or participating in collecting sponges. During the project period, we have worked with people at two different community colleges in Minnesota, one college in Minnesota, and three people at different institutions throughout the United States. Because of this funding through LCCMR, we have been able to help provide more information about the importance and distribution of freshwater sponges to the general public in Minnesota, scientists and teachers in Minnesota, and interested parties throughout the United States.

Lastly, as the project period is ending and have a full and robust dataset, we are also preparing multiple manuscripts related to this research. We are currently working with our undergraduate students to write an ecological manuscript focused on the distribution of freshwater sponges in Minnesota and their associated water chemistry at each location. We are also working on a manuscript to describe the new species of sponge identified in the state. We expect there will be additional manuscripts related to potential new species of sponge in Minnesota. We are also working on a manuscript to describe the chemical makeup of sponges that was identified from our analyses of the chemical extracts. Because of the lack of research on freshwater sponges, this research has been fruitful in producing publishable data.

VI. PROJECT BUDGET SUMMARY:

A. Preliminary ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$188,500	Project manager 1 month salary each year ($27,000); project partner 0.5 month salary first year, 1 month salary last two years ($25,000); project partner 0.5 month salary the first year, 1 month salary the last two years ($26,000); three undergraduate students full time in each summer ($40,455); 4 undergraduate students averaging 7.5 hours per week during each academic year ($33,120); Research Associate (summer term) position for 2 summer months during year 1 to perform water and sponge collections by SUBA diving. If needed, 3 summer months year 2 and 1 summer month year 3 ($31,800). An ANDI certified diver with solo diver and diver first aid certification to serve in a dive safety capacity ($4,500.00).
Equipment/Tools/Supplies:	$49,000	Supplies for sponge and water collections for ~100 samples and SCUBA air tank fills (Per sponge sample - $10 for tubes, $10 for glass and Nalgene bottles for water, $10 for proper storage of traveling with samples, $30 for filling SCUBA air tanks/collection = $6,000 total); Supplies for extraction, amplification, and sequencing of DNA from ~100 sponge samples (Per sponge sample - $20 for DNA extraction, $40 for amplification, and $100 for sequencing reactions = $16,000 total); Supplies for chemical analysis (columns, solvents and buffers, analytical standards, UV-visible quartz tubes; $12,000 total); LC/MS and GC/MS Instrument access at the Center for Mass Spectrometry and Proteomics at the UMNTC to perform chemical analyses (~120 samples at $100 = $12,000 total).
Capital Expenditures over $5,000:	$10,000	Lyophilizer (Freeze-dryer from Fisher Scientific -Labconco™ 7752040) - needed to remove water from sponge samples in order to perform extraction of organic substances.
Travel Expenses in MN:	$4,000	Mileage for PIs to travel to sampling sites to oversee collection of sponges and perform preliminary chemical analysis of sample sites.
Other: Shipping	$2,000	Shipping costs to send samples to UMNTC for DNA sequencing and chemical analysis.
Professional/Technical Service Contract	$4,500	Safety Technician - An ANDI certified diver with solo diver and dive first aid certification who will be present on all collection trips. The presence of a safety technician is recommended by dive professionals in all recreation and commercial dive operations. The safety technician will only serve in dive safety capacity.
TOTAL ENRTF BUDGET:	**$258,000**	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: One lyophilizer (freeze-dryer) will be purchased ($10,000; Labconco™ model no. 7752040) for removal of water from sponge samples in order to extract organic substances. Upon the completion of this project, the instrument will continue to be used by the University of Minnesota Crookston for similar projects and purposes. Currently, there is no such instrument available on campus nor at surrounding colleges or universities. Other groups who need such instrumentation will be able to utilize the equipment without charge. If the instrument is sold prior to the end of its useful life, proceeds from the sale will be paid back to the Environment and Natural Resources Trust Fund.

Total Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 7.0 FTE

Total 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			
In-Kind Support	$ 134,160	$	Indirect costs (waived)
State			
	$	$	
TOTAL OTHER FUNDS:	**$ 134,160**	**$**	

VII. PROJECT STRATEGY:

A. Project Partners:

Partners receiving ENRTF funding

- Venugopal Mukku*, (*Associate Professor), Anthony Schroeder (Assistant Professor), Timothy Dudley (Assistant Professor), University of Minnesota Crookston: $208,000 to extract and quantify contaminants from the collected sponges, perform morphological and molecular identification of collected of all freshwater sponges and integrate this research into the undergraduate programs at UMC. All University of Minnesota Crookston faculty receiving funding will also be supervising students.

- Karl Anderson, Research Associate (Summer Term), University of Minnesota Crookston. $31,800 to perform sponge and water collections in two summer months of year 1 via SCUBA diving, 3 summer months in year 2, and 1 summer month in year 3, but only after successful preliminary findings using SCUBA in year 1. Karl is SCUBA certified and has a Master's Degree in Environmental Ecology. A major goal of this project is to determine the distribution of freshwater sponges in Minnesota. However, peer-reviewers felt that most likely the freshwater sponges will be found in shallow water (1-2 meters). Although this may be true, because we want to determine the full potential distribution of freshwater sponges, we also want to determine if freshwater sponges occur at deeper depths in Minnesota's lakes, which has not previously been studied nor reported. With this in mind, the first summer will be used as preliminary studies to determine if freshwater sponges occur at depths greater than a few meters. If the first year finds freshwater sponges do occur at deeper locations, diving will occur in years 2 and 3. If sponges are not found at greater depths, we will forego SCUBA in years 2 and 3. If money is not spent

related to SCUBA diving collections in years 2 and 3, the funds will be returned to the ENRTF. This preliminary investigation approach provides a more efficient use of funds related to the project.

- **Partners NOT receiving ENRTF funding: N/A**

B. Project Impact and Long-term Strategy: The long-term goal of the proposed study is to provide an understanding of the distribution of the freshwater sponges found in Minnesota. This research is especially important because there is no current data regarding these essential aquatic organisms. Consequently, the potential of sponges to uptake pollutants results in bioaccumulation of pollutants which would be transferred to gamefish and ultimately humans. Results will be disseminated through scientific presentations, peer-reviewed publications, and presented to interested state agencies.
This project will provide opportunities for Minnesota students to receive training in relevant field and laboratory protocols, thereby ensuring a well-prepared science workforce.

C. Funding History:

Funding Source and Use of Funds	**Funding Timeframe**	**$ Amount**
Service contract to Anthony Schroeder funded through the ENRTF unrelated to the current proposal (053-B Eliminating Contaminants to Protect Endangered Native Fish/Mussels). Funds used to develop molecular and physiological endpoints in fish and mussels	7/01/2016 - 6/30/2019	$ 33,000
Vice Chancellor of Academic Affairs, University of Minnesota Crookston – Interdisciplinary grant to Anthony Schroeder and Timothy Dudley. Funds were used to perform initial collections and obtain preliminary data related to freshwater sponges in Minnesota	7/1/2016 – 6/30/2017	$ 20,000

VIII. REPORTING REQUIREMENTS:

- **The project is for 4 years, will begin on 07/01/17, and end on 06/30/21.**
- **Periodic project status update reports will be submitted February 1 and August 1 of each year.**
- **A final report and associated products will be submitted between June 30 and August 15, 2021**.

IX. VISUAL COMPONENT or MAP(S): See attached graphic.

X. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS:

A. Parcel List: N/A

B. Acquisition/Restoration Information: N/A

Environment and Natural Resources Trust Fund
M.L. 2017 Final Project Budget

Project Title: Mapping Taxonomy and Environmental Toxicology of Minnesota Freshwater Sponges
Legal Citation: M.L. 2017, Chp. 96, Sec. 2, Subd. 03m
Project Manager: Anthony Schroeder
Organization: University of Minnesota Crookston
M.L. 2017 ENRTF Appropriation: $ 258,000
Project Length and Completion Date: *4* Years, June 30, 2021
Date of Report: August 16, 2021

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget (03/15/2021)	Amount Spent	Activity 1 Balance	Revised Activity 2 Budget (03/15/2021)	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Collection and taxonomic identification of freshwater sponges sampled from the ten major basins and watersheds in Minnesota***			***Detection of contaminants present in collected freshwater sponges***			***Training undergraduate students in interdisciplinary research and dissemination of research findings to the appropriate stakeholders***				
Personnel (Wages and Benefits)	$84,000	$84,000	$0	$54,500	$54,500	$0	$50,000	$50,000	$0	$188,500	$0
Anthony Schroeder, Project Manager: $27,000 (66% salary, 34% benefits); 11% FTE each year.											
Venugopal Mukku, Senior Personnel: $25,000 (66% salary, 34% benefits); 5.5% FTE first year; 11% FTE for last 2 years.											
Timothy Dudley, Senior Personnel: $26,000 (66% salary, 34% benefits); 5.5% FTE first year, 11% FTE for last 2 years.											
4 Undergraduate Research Assistants during academic year: $37,520 (92.1% salary, 7.9% benefits). 2 students at 25% FTE (10 hours/week) each year; 2 students at 12.5% FTE (5 hours each week) each year											
3 Undergraduate Research Assistants during each summer: $40,455 (92.1% salary, 7.9% benefits). 3 at 100% FTE (3 months of summer) each year											
Karl Anderson, Research Associate (Summer Term): The summer term research associate is a certified diver with a Master's Degree with an emphasis in Environmental Ecology and will assist in sponge collections. The position will be a summer term position (no more than 75 days/summer). 2 summer months in year 1 ($21.34 per hour/8 hours per day/45 summer days = $10,000). 3 summer months in year 2, if needed ($21.34 per hour/8 hours per day/70 days =$15,200). 1 summer month in year 3, if needed ($21.34 per hour/8 hours per day/30 days=$6,600). 72.8% salary, 27.2% fringe benefits.											
Professional/Technical/Service Contracts (Amendment approved 09/25/2017):											

Safety Technician - An ANDI certified diver with solo diver and dive first aid certification who will be present on all collection trips. The presence of a safety technician is recommended by dive professionals in all recreation and commercial dive operations. The safety technician will only serve in dive safety capacity	$4,500.00	$4,500	$0							$4,500.00	$0.00
Equipment/Tools/Supplies											
Supplies for sponge and water collections (Tubes, Nalgene and glass bottles, supplies to store and travel with speciments, and SCUBA air tank fills) (~100 sponge and water samples/$60 per sample)	$6,000	$6,000	$0							$6,000.00	$0.00
DNA extraction kits to isolate DNA from collected sponges (~100 samples/$20 per sample)	$2,000	$2,000	$0							$2,000.00	$0.00
Reagents and supplies to perform DNA sequencing reactions (primers, Big Dye reagent, tubes) (~100 samples/$40 per sample)	$4,000	$4,000	$0							$4,000.00	$0.00
DNA sequencing performed by the Genomics Center, UMNTC). for sponge identification (~100 sponge samples/4 sequencing reactions per sample at $25 per reaction)	$10,000	$10,000	$0							$10,000.00	$0.00
General chromatographic and spectrophotometric supplies (columns, solvents and buffers, analytical standards, UV-visible quartz tubes)				$15,000	$12,000	$3,000				$15,000	$3,000
LC/MS and GC/MS instrument access. These analyses will be performed off-site (Center for Mass Spectrometry and Proteomics, UMNTC) and will be used to identify and quantify contaminants in the sponge samples (~120 samples/$100 per sample)				$12,000	$8,141	$3,859				$12,000	$3,859
Capital Expenditures Over $5,000											
Lyophilizer (Freeze-dryer from Fisher Scientific -Labconco™ 7752040) - needed to remove water from sponge samples in order to perform extraction of organic substances				$10,000	$10,000	$0				$10,000	$0
Travel expenses in Minnesota											
Mileage costs (59 cents per mile, 6000 miles total) for PIs to travel to sampling sites to oversee collection of sponges and perform preliminary chemical analysis of sample sites. 2 trips to each of the 10 watersheds and basins (10 round trips year 1 at a cost of $2000, 10 round trips year 2 at a cost of $2000) originating from UMC (Crookston).	$4,000	$4,000	$0							$4,000	$0
Other											
Shipping costs to send samples for DNA sequencing (400 sequencing reactions) and chemical analyses at UMNTC (120 samples)	$1,000	$1,000	$0	$1,000	$164	$836				$2,000	$836
Unallocated funds - funds moved from the contract for bid. Funds will be allocated only if needed to complete the project, otherwise funds will be returned to the ENRTF at the end of the project.	$0	$0	$0							$0	$0