M.L. 2016 Project Abstract
For the Period Ending June 30, 2019

PROJECT TITLE: Assessing Techniques for Eliminating Contaminants to Protect Native Fish and Mussels
PROJECT MANAGER: Kristine Wammer
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2016, Chp. 186, Sec. 2, Subd. 04d

APPROPRIATION AMOUNT: $287,000
AMOUNT SPENT: $271,441
AMOUNT REMAINING: $15,559

Sound bite of Project Outcomes and Results
This project assessed whether UV treatment will effectively remove toxicity attributable to commonly reported wastewater contaminants: polycyclic musks. Several adverse biological effects were observed for these contaminants, but only at concentrations higher than typically observed in Minnesota waters. These effects are mostly reduced or eliminated upon exposure to UV light.

Overall Project Outcome and Results
In 2009 the MPCA was directed by the legislature to monitor surface waters for endocrine disrupting compounds near wastewater treatment plants. In the resultant study, two of the most commonly detected compounds (tonalide and galaxolide) were polycyclic musks that are used as synthetic fragrances in a wide range of products. It has been demonstrated in mussels that musks can impair transporters involved in the first line of defense against toxicants. This is of great concern as 25 of Minnesota's 48 native mussel species are listed as endangered, threatened, or of special concern. Tonalide and galaxolide are also known to induce other types of toxicity (e.g. liver damage, DNA/genetic damage) and are suspected endocrine disruptors, meaning they can disrupt hormones and impair growth and reproduction, and are thus a potential threat to mussel and fish populations.

The goal of this project was to assess whether UV treatment of wastewater can effectively remove toxicity attributable to these compounds. When tonalide was exposed to sufficient UV light, it was eliminated and several photoproducts were formed. Biological assays were performed using tonalide and confirmed endocrine activity and inhibition of transporters as predicted, but only at high concentrations that would not be typically expected in Minnesota waters. Furthermore, biological effects were largely reduced or eliminated upon exposure to UV, suggesting photoproducts do not retain significant biological activity of the parent compound. Analysis of wastewater effluents reveals the presence of some parent tonalide and some photoproducts, supporting the prediction that higher UV doses than currently used may be required to completely remove tonalide. A major finding of this project is that galaxolide is much less stable in water than previously reported; it is unlikely to persist and be of concern in environmental waters. Therefore, overall this study suggests polycyclic musks are unlikely to be an imminent threat at the levels detected in Minnesota waters.

Project Results Use and Dissemination
Results from this project have, to date, been disseminated primarily via presentations given by undergraduate students from Gustavus and the University of St. Thomas. Locally, this included the 2019 "Scholars at the Capitol" event. Nationally, students have presented at American Chemical Society and Society for Environmental Toxicology and Chemistry meetings; internationally, a student presented at the International Symposium on

Liquid Phase Separations. Because the bulk of the substantive results have been finalized in the past few months, preparation of manuscripts for submission to peer-reviewed journals are forthcoming.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2016 Work Plan Final Report

Date of Report: August 31, 2019

Final Report

Date of Work Plan Approval: June 7, 2016

Project Completion Date: June 30, 2019

PROJECT TITLE: Assessing Techniques for Eliminating Contaminants to Protect Native Fish and Mussels

Project Manager: Kristine Wammer

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Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$287,000**
	Amount Spent:	**$271,441**
	Balance:	**$15,559**

Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 04d

Appropriation Language:

$287,000 the second year is from the trust fund to the commissioner of natural resources for an agreement with the University of St. Thomas to evaluate the use of ultraviolet treatment of wastewater to remove certain commonly detected wastewater contaminants, in order to reduce the contaminants' toxicity to native fish and mussels. This appropriation is available until June 30, 2019, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Assessing Technique for Eliminating Contaminants to Protect Native Fish and Mussels

II. PROJECT STATEMENT:

In 2009 the MPCA was directed by the legislature to monitor surface waters for endocrine disrupting compounds (EDCs) in the vicinity of at least 20 wastewater treatment plants (WWTPs); in the resultant study tonalide and galaxolide were detected in 84% and 96% of effluent samples respectively, as well as in many sediments downstream from WWTPs (30-60%). This project will determine whether (a) UV disinfection would effectively remove these contaminants prior to discharge into surface waters and (b) whether the products formed when the contaminants break down would still be of concern.

Although tonalide and galaxolide are among the most commonly detected contaminants of emerging concern (CECs) in Minnesota WWTP effluents, the effects of these high production volume chemicals and their byproducts on the quality of Minnesota drinking water and aquatic life remain largely unknown. Municipalities in various locations are considering costly modifications of existing wastewater treatment processes to enhance removal of such CECs to protect surface waters, many of which serve as sources of drinking water, without adequate understanding of whether such treatments are effective and/or necessary. UV treatment is commonly considered because it can be used simultaneously to improve chemical removal and disinfect wastewater.

Both contaminants to be studied have worldwide production volumes of over 6,000 tons per year and account for 90% of the US market for polycyclic musks, which are used as synthetic fragrances in a wide range of products. Musks can impair transporters involved in the first line of defense against toxicants, known as MXR/PXR defenses. These transporters are involved in substrate translocation across membranes and mediate cellular efflux of a variety of organic chemicals. If detoxification ability is impaired, organisms cannot effectively eliminate other toxic chemicals found in MN waters - this has been demonstrated in mussels. This is of great concern as 25 of Minnesota's 48 native mussel species are listed as endangered, threatened, or of special concern. Tonalide and galaxolide are also known to induce other types of toxicity (e.g. liver damage, DNA/genetic damage) and are suspected EDCs, meaning they can disrupt hormones and impair growth and reproduction, and are thus a potential threat to mussel and fish populations.

This project will assess whether UV treatment of wastewater will effectively remove toxicity attributable to these common wastewater contaminants, including assessing toxicity of products formed during UV treatment. UV treatment can be effective at reducing tonalide concentrations in effluent, but galaxolide is tougher to break down. It is of particular concern that most galaxolide degradation products have been classified as very persistent and/or toxic. Therefore, there is an urgent need to further our understanding of what is formed when these contaminants are broken down by UV light as it is very possible these UV products could also be an important unknown source of toxicity for endangered native mussels and fish in MN waters. This work will provide valuable insight into the ability of UV treatment to mitigate contribution of these contaminants to toxicity of wastewaters, in addition to identifying contaminants and products of particular interest for monitoring and further study and enabling municipalities to make better informed decisions about the need for treatment upgrades.

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of February 1, 2017:

Initial method development for measuring concentrations, effectively separating parent compounds and photoproducts, and performing in vitro toxicity assays has been successfully completed in the Wammer, Martinovic-Weigelt, and Stoll labs. The rate at which both polycyclic musks (tonalide and galaxolide) degrade under both simulated sunlight and UV-C light has been quantified, although UV-C degradation kinetics are more complex than anticipated and are the subject of ongoing experiments. Photoproduct characterization is underway, and several tonalide photoproducts have tentatively been identified. Initial in vitro toxicity testing has shown that neither musk compound exhibits overt cytotoxicity or androgenic activity. Estrogenic and antiestrogenic activity testing is under development. The only noteworthy challenge to date has been significant background signals for parent compounds and photoproducts, apparently due to lab contamination. The Stoll group has taken the lead on troubleshooting and developing protocols to overcome this challenge.

Project Status as of August 1, 2017:

Method development continues both on the analytical and biological assay portions of the project, and good progress has been made. We have significant preliminary results for kinetics and some photoproduct characterization, and work continues in these areas. A new analytical tool (GC-MS) has been added to complement the LC-MS experiments, which may allow characterization of additional photoproducts. A catastrophic instrument failure led to a temporary disruption of the plan to collect and analyze WWTP samples via LC-MS, so that portion of the project has been delayed from our original projections. However, a new instrument will be available beginning this fall so we anticipate getting back on track and should be caught up by this time next year.

Project Status as of February 1, 2018:

Method development has continued on both the analytical and biological assay portions of the progress, with many methods now ready or almost ready for use. Collection and analysis of WWTP and surface water samples will begin in late spring or early summer. Some in vitro assays have been conducted on both galaxolide and tonalide and have thus far shown no deleterious biological effects at concentrations relevant to Minnesota surface waters. Work is ongoing to prepare photoproduct mixtures for the various biological assays under development.

Project Status as of August 1, 2018:

Significant progress has been made in key areas, including determination of tonalide photolysis kinetics and generation of tonalide photoproduct mixtures for testing in biological assays. Biological assay development has progressed to the point where most experiments are ready to be performed. We are, however, requesting an amendment to move the project completion date by six months for two main reasons. First, we have struggled to get permission to sample effluents at wastewater treatment plants but hope to sample more extensively next summer assuming we can make progress on this over the next few months. Second, we have found that poor galaxolide stability in water is complicating our efforts to obtain some data; this is something that has not previously been reported in the literature and is requiring us to perform additional experiments prior to moving forward with further analysis of galaxolide or its photoproducts.

Amendment request withdrawn 10/15/18

Project Status as of February 1, 2019:

While galaxolide stability remains an issue, we have now quantified degradation rates and found them predictable enough that we can work with galaxolide as long as we use an adequate concentration of methanol or acetonitrile co-solvent. Testing of the ability of tonalide and its photoproducts to interfere with 48 nuclear receptors (these receptors are important for normal biological function) indicated that UV treatment either reduced the ability of tonalide to interact with these receptors or left it unchanged; there were no cases where UV enhanced interference with the receptors. Thus we conclude that UV treatment can be helpful in reducing toxicity of tonalide. Additional biological assays have been tested and are ready to go; procedures have been developed to produce the quantities of pure galaxolide required for the various biological assays. Those experiments should be completed in the new few months. We have still struggled to gain access to wastewater treatment plants, however; we really need to obtain access soon if we are to successfully analyze effluent samples prior to the end of the grant.

Overall Project Outcomes and Results:

In 2009 the MPCA was directed by the legislature to monitor surface waters for endocrine disrupting compounds near wastewater treatment plants. In the resultant study, two of the most commonly detected compounds (tonalide and galaxolide) were polycyclic musks that are used as synthetic fragrances in a wide range of products. It has been demonstrated in mussels that musks can impair transporters involved in the first line of defense against toxicants. This is of great concern as 25 of Minnesota's 48 native mussel species are listed as endangered, threatened, or of special concern. Tonalide and galaxolide are also known to induce other types of toxicity (e.g. liver damage, DNA/genetic damage) and are suspected endocrine disruptors, meaning they can disrupt hormones and impair growth and reproduction, and are thus a potential threat to mussel and fish populations.

The goal of this project was to assess whether UV treatment of wastewater can effectively remove toxicity attributable to these compounds. When tonalide was exposed to sufficient UV light, it was eliminated and several photoproducts were formed. Biological assays were performed using tonalide and confirmed endocrine activity and inhibition of transporters as predicted, but only at high concentrations that would not be typically expected in Minnesota waters. Furthermore, biological effects were largely reduced or eliminated upon exposure to UV, suggesting photoproducts do not retain significant biological activity of the parent compound. Analysis of wastewater effluents reveals the presence of some parent tonalide and some photoproducts, supporting the prediction that higher UV doses than currently used may be required to completely remove tonalide. A major finding of this project is that galaxolide is much less stable in water than previously reported; it is unlikely to persist and be of concern in environmental waters. Therefore, overall this study suggests polycyclic musks are unlikely to be an imminent threat at the levels detected in Minnesota waters.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Quantify removal of contaminants by UV treatment and measure toxicity and endocrine disrupting activity

Description:

Tonalide and galaxolide photolysis rates, quantum yields and extent of removal with UV treatment will be quantified in the laboratory to enable estimation of transformation efficiency during wastewater treatment. Mixtures of UV degradation products will be generated for toxicity testing, with a focus on adverse effects on fish and mussels. In situations where chemicals and their photoproducts are present in complex mixtures, biological analyses that can quantify total toxicological activity without knowledge of specific chemical composition can be used to streamline identification of chemicals/fractions responsible for the observed biological activity. The following battery of *in vitro* assays will be used to determine whether UV exposure can reduce/eliminate toxicity of galaxolide and tonalide by screening parent compounds and photoproduct mixtures/fractions:

- *Detoxification assays – test for impairment of organism's ability to eliminate contaminants*
    - If MXR toxicity is detected, we will conduct native fish and mussel tests to determine whether exposure to musks/their UV products can increase toxicity of common contaminants that normally co-occur in WWTP effluents with musks.
- *Endocrine toxicity assays - test for disruption of reproductive hormones (e.g., testosterone, estrogen)*
    - If endocrine cell toxicity is detected, we will conduct 48 h exposure of adult fathead minnows (an excellent model for MN natives) to the musks/photoproducts of interest and evaluate effects on expression of genes involved in endocrine function
- *General toxicity assays* – a series of widely recognized tests indicative of toxicity used for human and ecological hazard evaluation will be measured. Parent compounds and their photoproducts will be analyzed for approximately 90 different toxicity endpoints (including carcinogenesis, DNA damage, endocrine disruption, neurotoxicity etc.) using commercially available, cutting-edge techniques where living cells/proteins are exposed to water samples and screened for changes in biological activity that are indicative of toxic effects. If resources allow, other toxic pathways of interest (especially those that are

initiated at environmentally relevant concentrations) indicated by general toxicity assays will be evaluated.

If biological assays suggest that some of these UV degradation products are toxic they will be further characterized primarily via liquid chromatography coupled with Time-of-Flight mass spectrometry; see Activity 2 for a more detailed description of the analytical methods. If possible, active products will be isolated or, if available, purchased for individual compound toxicity testing.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 200,000
Amount Spent: $ 198,467
Balance: $ 1,533

Outcome	Completion Date
1. Measure photolysis rates and quantum yields of tonalide and galaxolide under UV light.	December 2017
2. Perform biological screening tests and, where appropriate, follow-up fish and mussel studies to determine if UV treatment can minimize toxicity to native fish and mussels.	January 2019
3. Identify toxic products formed during UV treatment	June 2019

Activity Status as of February 1, 2017:

Wammer and three undergraduate students: Analytical methods have been developed to monitor degradation of both galaxolide and tonalide by HPLC. In addition, a flash chromatography method was developed so that galaxolide parent compound can be purified from a significant diethylphthalate contaminant prior to use. A Rayonet-type photoreactor was obtained and set up so that UV-C photolysis experiments can be conducted. Work is ongoing to deal with background signals and laboratory contamination that may impact product identification and in vitro toxicity testing assays (see Activity 2 status for more details.)

Photolysis rates and quantum yields for both compounds have been successfully determined using simulated sunlight. Observed simulated sunlight rate constants are k_{obs} = 5.14 x 10^{-4} for tonalide and k_{obs} = 5.36 x 10^{-5} for galaxolide; sunlight quantum yields (the fraction of absorbed photons that result in transformation of a molecule) are 5.87 x 10^{-3} for tonalide and 1.48 x 10^{-3} for galaxolide. Therefore, although somewhat slow, degradation for both compounds appears to be rapid enough for sunlight photolysis to be an environmentally relevant process. As a result, work in Activity 2 in the future will include looking for major photoproducts in receiving waters of both UV- and non-UV treated wastewaters. Observed rate constants for both compounds have also been determined under UV-C radiation: k_{obs} = 2.30 x 10^{-2} s^{-1} for tonalide and k_{obs} = 9.66 x 10^{-3} s^{-1} for galaxolide. These rates are higher for both compounds, as expected due to better spectral overlap with the UV-C lamps. Work to determine UV-C quantum yields using the iodide-iodate actinometer is in process. It should be noted that all rates determined to date assume first order kinetics, but recent experiments have shown that under certain conditions compound degradation appears to follow some sort of biphasic rate so more work is necessary to unravel this potential complication. Work is also currently being done in collaboration with the Stoll group to tentatively identify major photoproducts for both musk compounds. Several likely tonalide products have been found to match expected products from the literature. However, several more products for both compounds have yet to be characterized; this effort is complicated by the fact that photoproduct suites are dependent on both light source and intensity.

Martinovic-Weigelt and three undergraduate students: Two in vitro toxicity testing assays (cytotoxicity and androgenic/anti-androgenic endocrine activity assays) were set up. Both galaxolide and tonalide were tested in cytotoxicity and endocrine assays. Neither chemical exhibited overt cytotoxicity. Neither was androgenic even when tested at the concentrations higher than those typically measured in the environment. Findings for tonalide are congruent with published data obtained using the same cell line, whereas galaxolide data are not available. A systematic search of high-throughput toxicity data depositories established that there is a lack of

comprehensive mechanistic toxicity assessment for galaxolide, whereas such data was available for tonalide (iCSS ToxCast Dashboard version 1.0: https://actor.epa.gov/dashboard/#Assays). Finding that tonalide affected estrogen receptor (ER) function and hypoxia inducing factor (HIF1a) activity, and that these two targets were amongst 10 most sensitive molecular targets (consistently activated at < 5 uM concentrations), is of special concern as inhibition and/or inappropriate timing of activation of these molecular targets has been linked to impaired reproductive function in fish. To test whether interaction with these molecular targets has adverse effects on reproduction we transferred/developed laboratory methods (gene expression assays for HIF-responsive and ER-responsive genes linked to reproduction). We also conceptualized a toxicity pathway for HIF (https://aopwiki.org/wiki/index.php/Aop:123) to guide our future in vivo experiments that will establish whether interaction with these molecular targets can lead to adverse impacts on fish reproduction. Martinovic-Weigelt and students also set up and developed a protocol for preparation and extraction of the environmental samples for testing of the parents (tonalide and galaxolide) and their UV products in the general toxicity assays. This group is currently setting up an estrogenic activity assay to test galaxolide and tonalide for estrogenic and antiestrogenic activity.

Martinovic-Weigelt lab's work to date indicates that there is a need to assess toxicity of both parent compounds and their UV products to fish and mussels, and that these contaminants have a potential to impact reproduction via several distinct/yet complementary molecular mechanisms, including endocrine disruption.

Activity Status as of August 1, 2017:

Wammer and three undergraduate students: AHTN continues to exhibit biphasic degradation under 254 nm UV-C irradiation. However, degradation seems to be dependent on lamp strength. With an irradiance of about 40 W/m^2, first order degradation is observed as expected, whereas with 10 W/m^2 AHTN does not degrade at a constant rate. More trials will be performed to better understand the mechanism of degradation for AHTN. An effective separation method for HHCB from diethyl phthalate has been established. HHCB arrives in a roughly 50% mixture with diethyl phthalate, and must be separated since diethyl phthalate also undergoes photodegration. Initially we assumed that our separation method, which consists of using a Biotage Isolera flash chromatography instrument, was not completely effective in separating diethyl phthalate. However, GC results show no trace of diethyl phthalate after separation (although it does appear that several isomers and/or other contaminants remain in the purified sample.) HHCB degradation, which was initially thought to be second order from residual diethyl phthalate, must be degrading in a similar biphasic nature. Further work with the KI/KIO_3 actinometer shows promise for determining quantum yields for AHTN and HHCB exposed to UV-C irradiation. Quantum yield equations using the KI/KIO_3 actinometer have been found in the literature, and unique instrument variables such as the reflectivity coefficient of UV light have been determined. Solubility and concentration issues have been present throughout work with the actinometer, but the change from anhydrous borax to borax-decahydrate should aid in greater solubility. Work on quantum yield and kinetics quantification will continue into the fall.

In collaboration with Tony Borgerding (St. Thomas), we have been working to develop new methods for characterizing photoproducts due to temporary instrument problems at Gustavus (see Activity 2 for more details.) Tonalide and galaxolide, as well as their previously identified photoproducts, are sufficiently volatile for analysis by gas chromatography. We therefore developed methods to analyze aqueous solutions of these compounds using gas chromatographic mass spectrometry (GC-MS). Using GC-MS allows the use of solid phase microextraction (SPME), a simple and efficient way to improve sensitivity by orders of magnitude. This enhanced sensitivity may result in our ability to identify additional photoproducts that were too low in concentration to be measured by HPLC methods. Electron impact (EI) ionization (the ionization source used in GC-MS) offers complimentary structural information to electrospray ionization (ESI, used in HPLC-MS) because it is based on radical cations as opposed to ions formed by protonation of molecules. Our initial results this summer using GC-MS to analyze aqueous solutions of tonalide and galaxolide solutions that were photolyzed matched many of the results we had previously observed using HPLC, and the EI-MS spectra matched several of

the photoproducts reported in the literature and in our preliminary experiments using ESI-MS. We have developed studies to optimize the SPME sample prep in a way that is sensitive and reproducible. One concern moving forward is that tonalide and galaxolide show up in blanks and are hard to remove from the GC injection port after initial analyses. We are working on different geometries of injection ports, as well as QA/QC techniques to assure that this problem does not compromise the data we obtain using this technique.

Finally, we have been working on sample preparation for the biological assays being developed by the Martinovic-Weigelt and Schroeder labs (described below). Photolysis must be performed in water, but assay protocol requires samples to be dissolved in DMSO. Rotovapping has proven to be unsuccessful for retaining acceptable yields of parent compounds, so solid phase extraction (SPE) methods are being developed currently for parent compounds and will be subsequently tested using photoproduct mixtures.

Martinovic-Weigelt and two undergraduate students; Schroeder: Most of the effort (Feb –Aug, 2017) was on the method optimization, development and training of students. Three main efforts were underway: 1) Martinovic and Schroeder labs identified and optimized a suite of in vitro assays that can be used to test additional types of endocrine activity of the musks and their photoproducts, 2) Martinovic lab optimized the sample preparation process for future in vitro, high-throughput analyses, 3) Schroeder lab developed a putative adverse outcome pathway (AOP) to guide risk assessment for musks. First, bioinformatics analyses were conducted to identify applicable orthologs. The AOP posits that inhibition of ABCB4 (orthology of MXR and ABCB1) will lead to a decrease in ABCB4 Transporters and a change in ATPase activity. This would result in increased concentrations of some contaminants (concurring with musks that utilize ABCB4) and lead to decreased population trajectory due to increased mortality. It was also established that inhibition of ABCB4 transporters by musks may lead to changes in blood-brain barrier permeability leading to neurological effects and ultimately death.

Activity Status as of February 1, 2018:

Wammer and one undergraduate student: Primary efforts in the Wammer lab this fall focused on development of a solid phase extraction method to concentrate tonalide and galaxolide photoproducts and get them into compatible solvents for the various biological assays under development, some of which are ready for use. This has proven to be a challenge, and the Stoll group is now working on this piece as well in the hopes that we can soon provide products for testing to the Martinovic-Weigelt and Schroeder labs. Once this task is completed, focus will shift back to photolysis kinetics. In addition, we are beginning to plan for sampling in early summer, so that we can take advantage of the methods under development in the Stoll lab to measure musks in wastewater-impacted surface waters.

Martinovic-Weigelt and three undergraduate students: Two in vitro toxicity testing assays (cytotoxicity and endocrine (estrogenic) activity assays were set up. Both galaxolide and tonalide were tested in cytotoxicity and estrogen assay. Neither chemical exhibited overt cytotoxicity. Neither was estrogenic when tested at the concentrations typically measured in the environment, or at the highest tested concentration (1 umol/L; circa 1000 times higher than typical concentrations found in the polluted lakes and streams). A systematic search of toxicity data depositories established that there is a lack of comprehensive mechanistic toxicity assessment for galaxolide, whereas such data was available for tonalide (iCSS ToxCast Dashboard). These studies show that tonalide can stimulate estrogen receptor (ER), but typically at concentrations above 1umol/L. This agrees with our data that also suggest that high concentrations of tonalide are needed to induce estrogenic effects. Typically, such concentrations are not found in MN streams.

Because musks have been shown to interact with the efflux proteins that utilize a lot of energy, we acquired instrumentation and developed methodology that can assess whether musks (and other chemicals that musks typically co-occur with in MN) can alter energy expenditure in human and fish cells, and fish embryos. Effects on the embryos are of particular concern because embryonic development consists of a set of highly coordinated

processes, and bioenergetic perturbation by chemicals could result in abnormal development. Our pilot data indicate that exposure of human cells to the musks galaxolide and tonalide (at concentrations found in polluted MN waters - circa 1 nmol/L) does not alter oxygen consumption or metabolic potential of these cells. Embryo studies are planned for the future.

Schroeder and two undergraduate students: The Schroeder lab has been optimizing the enzyme-linked immunosorbent assay (ELISA) for measuring the enzyme activity of peroxisomal beta-oxidation enzyme acyl-CoA oxidase (AOX). The assay has been optimized for measuring AOX activity in fathead minnow livers. The Schroeder lab is also currently working on optimizing the assay for measuring AOX activity in fathead minnow embryos.

The Schroeder lab has also developed and optimized gene expression assays using quantitative polymerase chain reaction (qPCR). The genes being evaluated through the qPCR assays include genes known to be regulated by PPAR activators, ABC transporters, and the reference gene 18s rRNA. The DNA sequences for each gene were extracted from the publically available fathead minnow genome (https://www.setac.org/page/fhmgenome). qPCR primers that spanned multiple exons were developed and ordered for each gene. The primers spanning multiple exons were necessary to limit the possibility of amplifying any potential genomic DNA that may be within the sample and obtaining erroneous expression results. Because of cost considerations, each assay was developed to use Sybr Green dye for measuring the gene expression, rather than the initially proposed TaqMan probes. Each qPCR assay was optimized using RNA extracted from livers of adult fathead minnows. In the near future, the Schroeder lab will be working to optimize the qPCR assays for fathead minnow embryos as well.

Activity Status as of August 1, 2018:

Primary efforts in the Wammer lab over this time period focused on photolysis kinetics. Because the actinometer previously in use (iodide/iodate) was presenting some methodological challenges, we switched to using atrazine as an actinometer. We now have reliable kinetic data for tonalide, including quantum yield. Due to problems with galaxolide stability that are still being resolved (discussed further below), galaxolide kinetic data will have to be finalized subsequent to these other experiments. Four students worked on this project for some period of time since the last status update (Brady Anderson, Emily Stickney, Ja Eon Cho, and Carlee Heiling), three supported by LCCMR funds, and one supported by internal research funds from the University of St. Thomas.

The Stoll group has worked to produce and characterize tonalide photoproducts in quantities sufficient for biological assays. These photoproduct mixtures have been provided to be sent off for biological assays by Attagene. We have also worked toward preparing photoproduct mixtures of galaxolide for biological testing, however this has proved to be much more difficult. After extensive characterization we have determined that: 1) commercially available galaxolide materials are full of impurities, and 2) when the putative parent compound is actually isolated, it degrades/transforms rapidly in water (minutes). These facts, which have not been described previously in the literature, make study of the behavior of the parent compound and photoproducts very challenging. Two Gustavus undergraduate students (Amy Crawford and Devin Makey) were involved in this work in June and July – one supported by LCCMR funds, and one supported by internal research funds from Gustavus Adolphus College.

Schroeder and two undergraduate students (Dalton Javner and Michaela Lano): The Schroeder lab has optimized the enzyme-linked immunosorbent assay (ELISA) for measuring the enzyme activity of AOX in fathead minnow embryos and larva by performing a time course experiment. Embryos that were 24, 48, 72, and 96 hours post fertilization (hpf) and 3-days post hatch larva were used for optimizing the ELISA process. The ELISA results showed that AOX protein expression is very low at 24 and 48 hpf (barely above the background of the assay), while the protein expression was significantly increased at 72 hpf and continued to increase with developmental age. The ELISA results suggest that any *in vivo* exposure during embryonic development with musks should not

occur prior to 48 hpf and should be closer to 72 hpf. This result is supported in that liver differentiation is at stage 27 (60 hpf) of fathead minnow development (Devlin et al., 1996).

The Schroeder lab has also been optimizing the qPCR assays for fathead minnow embryos and larva. The time points evaluated followed the same time course experiment of the ELISA above (24, 48, 72, 96 hpf and 3-days post hatch). The genes being evaluated are still those known to be regulated by PPAR activators, ABC transporters, and the reference gene. The majority of the genes could be optimized for these time points, except PPAR gamma and fatty acid binding protein 1 (Fabp1). These genes show two peaks in the dissociation curve, suggesting a single product was not being produced for these genes. Therefore, new qPCR primers were developed using sequences from the fathead minnow genome browser. We are currently using these primers and to prepare standards for each gene and optimize each qPCR assay. For the other optimized genes, expression of each gene was measured at each embryonic and larval time-point. The qPCR showed similar results to the AOX ELISA, with very low gene expression (Ct < 32) for all genes measured in the 24 and 48 hpf samples. The expression increased significantly for each gene by 72 hours and continued to increase through the 3-day post hatch larvae. The gene expression results support the finding that any embryonic exposure to musks should occur after 48 hpf and that larval exposures would also be acceptable.

Activity Status as of February 1, 2019:

The Schroeder lab and one undergraduate student have been able to test the new qPCR primers for PPAR gamma and fatty acid binding protein (Fabp1). The new primers produced a single gene product in the qPCR and a set of standards for measuring the gene expression have been prepared. All of the qPCR assays for the genes that we hypothesized would be impacted by exposure to musks and their photoproducts, including PPAR activators and ABC transporters, have now been prepared and optimized for measuring by qPCR. Also, the ELISA for measuring the effects of the musk on specific protein expression has been optimized. All of these will be used in the upcoming months as a part of a larger study to measure the impacts of the musks on fish.

The Martinović-Weigelt team developed and optimized detoxification assays for work with MN native mussels. Gill tissues from *Elliptio* species were successfully used to evaluate the potential of tonalide to affect the ability of mussel gills to efflux ("expel") foreign chemical Rhodamine B. A short-term exposure to 10 µM tonalide reduced the ability of mussel gill to efflux foreign chemicals. This indicates that presence of the musks in wastewater and surface waters (at high concentrations) may enhance accumulation of other contaminants. The next step is to evaluate toxicity of tonalide photoproducts and galaxolide in this assay. We also evaluated the potential of tonalide and its photoproducts to interact with 48 different nuclear receptors representing a variety of biological processes. These analyses indicated that both 5 µM tonalide and 5 µM tonalide UV photoproduct interacted with seven nuclear receptors. Particularly affected were retinoic acid receptor alpha and beta, estrogen receptor alpha, pregnane X receptor, and NURR1 (upregulated more than 1.5 times relative to solvent controls). Interaction with these receptors indicate that these chemicals have a potential to affect the endocrine system and that they can activate contaminant-sensing receptors that help with degradation of the foreign chemicals. The activation of the receptor related protein NURR1 is of concern because NURR1 plays a key role in the maintenance of the dopaminergic system of the brain; mutations/dysregulation in this gene have been associated with a variety of diseases including arthritis and Parkinson's disease. Some positive news is that UV treatment either reduced ability of tonalide to interact with these receptors or left it unchanged; there were no cases where UV enhanced interference with the receptors. Thus we conclude that UV treatment can be helpful in reducing tonalide's toxicity.

The Wammer lab has been focusing on work with galaxolide over this time period. Due to the previously described issues with galaxolide stability in water, experiments were performed to determine galaxolide stability with a range of co-solvent concentrations. This work has been completed and the quantum yield experiments are now under way and will be completed soon.

Final Report Summary:

UV quantum yield determination and photoproduct characterization

Determining the importance of UV photolysis for transformation of tonalide and galaxolide presented many unanticipated methodological challenges throughout this project, but this work has now been successfully completed. Initial attempts to measure tonalide kinetics and quantum yield reliably were hampered by inconsistencies with the chosen actinometer with lamp intensity. Switching to atrazine, whose use as an actinometer for studies of environmental contaminant transformation by UV photolysis was published during the project period, enabled the measurement of reliable kinetics and a quantum yield of 0.056 $\pm$ 0.011. Three major photoproducts were characterized for tonalide, including molecular mass for all three and putative structures for two based on literature precedent. Mixtures of these photoproducts were generated, concentrated, and prepared for use in bioassays. Because toxicity assays showed no evidence of retaining significant biological activity relative to parent tonalide there was no need to isolate or obtain individual products for future testing. Discovery of galaxolide's lack of stability in water (described under Activity 2) presented additional challenges and also meant that it is unlikely to be of interest/concern in environmental systems. While instability in pure water meant there was no way to measure a quantum yield (galaxolide degradation is so rapid that photolysis can't compete) a quantum yield of 0.08 $\pm$ 0.01 was successfully measured in aqueous solutions with acetonitrile present as a co-solvent at 5%.

Screening toxic mechanisms of musks with high throughput in vitro toxicity assays

Because galaxolide photoproducts were very unstable and thus unlikely to be of importance, biological analyses of the UV effectiveness focused on tonalide and tonalide UV photoproducts. Focusing on tonalide allowed us to conduct additional types of toxicity screening assays (circa 50 more than initially planned). A series of widely recognized tests indicative of toxicity used for human and ecological hazard evaluation was conducted to determine whether UV treatment affected toxicity of the chemicals. Parent compounds and their photoproducts were analyzed for approximately **140 molecular toxicity endpoints** (including 48 human nuclear receptors, and a variety of molecules associated with carcinogenesis, DNA damage, endocrine disruption, neurotoxicity etc.) using cutting-edge techniques where living cells/proteins are exposed to water samples and screened for changes in biological activity that are indicative of potential toxic effects (www.attagene.com). These new, innovative methods can quickly and efficiently screen samples for toxicity and inform evaluation of their hazard to human and ecosystem health.

Molecular targets that were activated by 10 uM of tonalide are listed below:

1. Assay set 1 (CIS - 46 targets tested) included: pregnane X receptor (PXR)
2. Assay set 2 (TRANS 1- 24 targets tested): estrogen receptor alpha (ERa), pregnane X receptor (PXR), Retinoid X receptor-b (RXRb), Nuclear receptor related 1 (NURR1)
3. Assay set 3 (TRANS 2- 24 targets tested): RP, Retinoid X receptor-b (RXRg)
4. Assay set 4 (GPCR – 24 targets tested): Adenosine A2b receptor (ADORAB2), Adrenergic Receptor - beta 1 (ADRB1), and glucagon receptor (GCGR).

Tonalide photoproducts (TUV), generated from 10 uM tonalide (T) **exhibited toxicity lower** than parent tonalide (10uM) for the below listed targets:
Assay set 1 (CIS -52 targets tested): none
Assay set 2 (TRANS 1- 29 targets tested): ERa, RXRb, NURR1
Assay set 3 (TRANS 2- 29 targets tested): RP, RXRg
Assay set 4 (GPCR – 30 targets tested): ADORAB2, GCGR

Tonalide photoproducts (generated from 10 uM T) **exhibited toxicity comparable or higher** to that of parent tonalide T for the below listed targets:
Assay set 1 (CIS -52 targets tested): PXR
Assay set 2 (TRANS 1- 29 targets tested): PXR
Assay set 3 (TRANS 2- 29 targets tested): none
Assay set 4 (GPCR – 30 targets tested): ADRB1

The data described above indicates that tonalide photoproducts exhibit biological activity, and that i**n most of the cases the activity of tonalide is reduced by UV treatment** (with the exception of PXR and ADRB1 – their activation is not lowered upon UV treatment.)

Activation of the PXR might be a sign of chemical exposure and metabolism. PXR plays a role in xenobiotic (foreign chemical) sensing and induces metabolism/degradation of xenobiotics. Thus, induction of PXR should not be assumed to lead to adverse outcomes. Activation of **ERa** could have **implications for reproductive health of aquatic organisms**; it has been associated with endocrine disruption and reproductive malfunction. RXR play a role in mediating effects of retinoic acid which serves as a binding partner to other nuclear receptors, including PPARs, liver X receptors (LXRs) and vitamin D receptors (VDRs). Some of the dimers it forms (PPARα–RXR) regulate the expression of genes that regulate mitochondrial and peroxisomal β-oxidation and lipoprotein metabolism. Furthermore, **inappropriate activation of RXR could disrupt appropriate development.** Activation of adrenergic receptor - beta 1 (ADRB1) is likely to affect heart function and metabolism (lipolysis). The implication of activation of adenosine receptors (e.g., ADORAB2) should be investigated further; they have been indicated to play a role in mediating the development and hypoxic responses in fish.

It is important to note that none of the above targets were activated when tonalide and tonalide photoproducts were tested at lower concentration (i.e., 0.1 uM). While we successfully identified likely molecular mechanisms of action for T and TUV, organismal effects should be evaluated further to elucidate hazards in vivo and upon exposure to environmentally relevant concentrations.

Endocrine activity assays - test for the interaction of musks with steroid receptors that mediate reproductive function

Because nuclear receptors may differ in sensitivity and specificity across species, we also tested effects of tonalide and tonalide photoproducts (at three concentrations 10, 1 and 0.1 uM and three UV exposure lengths - 30, 60 and 120s) on nuclear receptors of several other species relevant to ecotoxicological risk assessment. Tests were performed as follows: 1. Fish (zebrafish), frog (Xenopus), turtle (painted turtle), bird (chicken) for **estrogen and androgen receptors** (ER, AR), 2. Fish, frog, and turtle for **thyroid receptors** (TR), and 3. fish for peroxisome proliferator activated receptor gamma (**PPARg**). Chemicals are reported as active if they activated molecular targets more than 1.5-fold relative to the controls. We confirmed prior findings (generated with human cell-based cell assays) of estrogenicity of T and TUV, and the lack of their androgenicity. Fish, frog, turtle and chicken receptor assays indicated that **T and TUV can activate estrogen receptors** in these species, with fish being the least sensitive. No effects on TR were observed in any of the species tested. None of the receptors were activated by lowest concentrations of T and TUV tested (0.1uM). **As hypothesized we found that the UV treatment typically reduced and/or eliminated estrogenic activity.** One exception to this was the observation that biological activity in the turtle ERa assay stayed the same (in parent vs. 120s TUV).

Chemosensitization tests with fish and mollusks

Multixenobiotic resistance (MXR) efflux competitive substrate/transporter inhibition assays – **a test for impairment of organism's ability to eliminate contaminants** – were performed; the fluorescent dye rhodamine B was used as an indicator of efflux transporter activity. Inhibition of transporter activity by a test compound was indicated by increased fluorescence due to higher accumulation of rhodamine B in the cells. Experiments were performed with native, North American mussel (*Elliptio complanata*); gill tissue, which shows high efflux transporter activity was used. **Tonalide, at a concentration of 10 uM** (concentration much higher than that observed in the MN surface waters)**, caused a significant increase in rhodamine B accumulation in *E. complanata* gill tissue indicating impairment of the ability to eliminate contaminants.** Other concentration tested (as low as 0.1 uM) did not increase rhodamine B accumulation/ efflux inhibition. Similar findings were obtained in the larval fish (*Pimephales promelas* – fathead minnow) experiments. The effect was only observed at the highest concentration tested (10 uM); the **effect was only significant for tonalide; tonalide photoproducts did not impair efflux even when tested at the highest concentration (10 uM, 120 s UV).**

We also conducted native **fish tests to determine whether exposure to musks/their UV products increases toxicity of a common group of contaminants** - phthalates. Phthalates were selected as test compounds because: 1) they commonly co-occur with musks in the WWTP effluents, 2) they are subject to removal by transporters involved in

MXR/MDR responses, 3) biomarkers of phthalate effects (i.e., peroxisome proliferation) are well characterized and easily measurable. It is well known that phthalates are peroxisome proliferators in aquatic organisms including mussels. Peroxisome proliferators comprise a heterogeneous group of compounds that typically act via activation of peroxisome proliferator-activated receptors (PPARs); many are known for their ability to cause liver cancer. The concentrations we used were environmentally relevant to MN streams.

To test whether polycyclic musk compounds can increase the toxicity of common contaminants, specifically phthalates, through chemosensitization we performed a 24 hour exposure with four-day old fathead minnow (*Pimephales promelas*) larva. The larva were exposed to either DMSO controls, diallyl phthalate, 10 nM tonalide (polycyclic musk), or 10 nM tonalide with diallyl phthalate. We used very low concentrations of musks (nM) to emulate conditions in MN surface waters. As noted above, phthalates activate PPARs. Therefore, the in vivo assays by the Schroeder group focused on activation of PPAR gamma protein and gene expression. Protein (ACOX1) and gene expression of genes know to be regulated by PPAR activators was also examined. The protein expression was measured using protein specific ELISA and the gene expression was measured using quantitative PCR using previously designed gene-specific primers. The protein expression was determined based on absorbance readings compared to protein specific standards. The relative gene expression was determined using the delta delta Ct method, with the ribosomal protein 8 gene as the control, to determine the biological impacts of the treatments compared to the DMSO controls.

An examination of the direct effect of the treatments on PPAR gamma protein expression showed there was not a significant effect of the exposure to tonalide or its photoproduct on the protein. Similarly, there was **not a significant effect of the treatments on ACOX1 protein expression**.

Because impacts on protein expression can take longer to observe, we also measured mRNA expression of PPAR gamma and other genes know to be regulated by PPAR activation. **There was no significant direct impact of the diallyl phthalate, tonalide, or the mixture of the two chemicals on PPAR gamma mRNA expression** compared to the DMSO control. **The tonalide treatment caused a significant increase in glycerol kinase mRNA expression** compared to the DMSO controls. There were no other significant impacts of the treatments on mRNA expression of the other genes known to be regulated by PPAR activation.

Overall, the **results do not support the hypothesis that the exposure to low, environmentally-relevant concentrations of musks lead to chemosensitization to phthalates**. Phthalates are known to be activators of PPAR; however, we did not observe a significant impact of the phthalates on PPAR protein or gene expression. The fact that there was no significant impact of the treatments on PPAR could also explain why there was little to no impact on mRNA or protein expression of genes known to be regulated by PPAR activation. It has been reported that peroxisome proliferation can occur very rapidly; however, it is possible that 24 hours was not sufficient exposure time to cause the activation of PPAR in the larvae. This could suggest that a 48 hour or longer exposure may be needed to see an impact of the treatments on PPAR activation to observe the potential chemosensitization effect by musks. There is also a possibility that the larvae are less sensitive to the impacts of the phthalates and musks; however, studies would suggest that early life-stage organisms are sensitive to these types of compounds.

Because there were no significant effects on mussels efflux proteins at environmental concentrations, and no effects on any of the fish endpoints (even at high concentrations) we did not conduct additional experiment with mussels; this minimized use of rare native mussels. Instead we conducted additional experiments that targeted additional/alternative mechanisms of action.

Additional/alternative tests with fish

We identified additional endpoints/targets for fish testing. We focused on the mitochondrial function endpoints because mitochondrial toxicity is a common mechanism of toxicity for many chemicals and it can be linked to adverse organismal and population level outcomes relevant to managers (i.e., energetic demand/metabolism/growth). Since musks inhibit efflux transporter proteins (function of which is energetically costly) they might also affect metabolism (i.e., oxygen consumption). To evaluate effects on the mitochondrial function we measured oxygen consumption rates of cells exposed to tonalide and its photoproduct (10 and 1 uM). We measured basal respiration (cells “at rest”)

and respiration when cells were stressed with a series of well-known mitochondrial stressors (to estimate spare capacity – ability to mobilize reserves in a stressful environment). **There was no statistically significant difference in the mitochondrial basal respiration or the spare capacity** in cells exposed to tonalide or its photoproducts (human and rainbow trout cell lines were used). Effects of musks on the respiratory costs of xenobiotic efflux mediated by ABC transporters remain of interest. Future experiments should include in vivo models.

ACTIVITY 2: Quantify contaminants and their UV products in municipal wastewater

Description:
Effluents from wastewater treatment plants with and without UV disinfection will be analyzed for the presence of the two target contaminants and the products formed when they undergo degradation by UV light. Professor Stoll's laboratory at Gustavus is equipped with state-of-the-art equipment that enables: 1) separation of a complex sample such as WWTP effluent and 2) identification of unknown compounds and quantitation of compounds of interest. This instrumentation will be especially useful to this project because it enables the identification of most products formed after UV treatment, and the measurement of very low concentrations of target compounds and products (akin to finding the 'needle in the haystack'). The Stoll group has extensive experience developing analyses for complex matrices.

In the first phase aimed at detection of the target musks and their known UV photoproducts, we will use online Solid-Phase Extraction (SPE) coupled with two-dimensional high performance liquid chromatography (2D-LC) with mass spectrometric detection. The high resolving power of 2D-LC will be especially valuable to the quantitation of photoproducts for which stable isotope labeled internal standards are not available, because of the mitigation of matrix effects that results from higher resolution of the sample constituents. This approach will also be used in the final phase of the work aimed at quantitation of photoproducts in WWTP effluent that have demonstrated endocrine activity or toxicity in the course of this project.

For the identification of additional UV photoproducts in WWTP effluent we will use comprehensive 2D-LC coupled with Time-of-Flight mass spectrometry to establish putative identities of the photoproducts using accurate mass measurements. These identities will be verified through retention time matching between authentic standards and the peaks observed in the mixture of transformation products.

Summary Budget Information for Activity 2:

ENRTF Budget: $ 87,000
Amount Spent: $ 72,974
Balance: $ 14,026

Outcome	Completion Date
1. Determine concentrations of target compounds and known UV products in at least six WWTP effluents and WWTP-impacted sites at least twice per year. Sites will be chosen to overlap significantly with those being studied in existing LCCMR-funded mussel-related work (e.g. Minnesota River basin, St. Croix River); the PI of the other study (Kozarek) will share sampling plans and field site locations.	June 2017
2. Identify additional UV degradation products in WWTP effluents based on major products observed during laboratory UV treatment studies.	June 2018
3. Measure concentrations of products determined to have endocrine activity or toxicity based on work carried out during this project.	June 2019

Activity Status as of February 1, 2017:

To date we have focused on developing a method for online-SPE coupled with one dimension of LC separation and Time-of-Flight (TOF) mass spectrometric (MS) detection. Arriving at suitable extraction, separation, and detection conditions has been very straightforward for standard solutions at high concentrations. The big

challenge so far has been in developing strategies to reduce background signals and contamination of analytical samples by musk parent compounds and degradants that are present in the laboratory environment. We now have a robust strategy to mitigate this contamination when analyzing intermediate volumes (e.g., 1 mL) of sample. However, moving to larger sample volumes requires different instrument components, and we are finding that some instrument components are significant sources of analyte carryover from one analysis to the next. We will be working with the instrument manufacturer to resolve this problem. Tentative identifications of the UV photoproducts of tonalide has been straightforward, by comparison of measured accurate masses to the exact masses of known photoproducts described in the literature.

Activity Status as of August 1, 2017:

Progress on this activity has been very slow since February. This has been the result of other project demands on some of the instrumentation used in this work, and then a catastrophic failure of the Time-of-Flight (TOF) mass spectrometer (MS) instrument in early April. We expect that the TOF-MS will be replaced with a new instrument in August, and that we will be able to get back on track with work on this activity relatively quickly. While we wait for the new TOF-MS to be installed, an undergraduate student in the Stoll Laboratory has been working on developing a predictive model that will help make informed choices of columns to use in two-dimensional liquid chromatography (2D-LC) separations of various samples. We expect that this model will ultimately be useful when we implement 2D-LC separations of the wastewater samples that we will study as part of this activity.

Activity Status as of February 1, 2018:

We have made substantial progress on this activity since August. Late in August a new TOF-MS instrument was installed in the Stoll Lab, followed by a new GC-MS, and then a QTOF-MS in late December. These instruments are all supporting the development of analytical methods that can be used for determination of tonalide and galaxolide and their photoproducts in WWTP samples, surface water, fish tissue, and musks photolyzed in the laboratory. Although there are several published methods for sample pretreatment for these different matrices, we have not yet found one that performs as reported with respect to recovery of the target analytes. Following the identification of the major photoproducts of tonalide and galaxolide by QTOF-MS, we will be able to finalize the method we will use for analysis of WWTP and surface water samples in the spring of 2018. One undergraduate student was involved in this work in December.

Activity Status as of August 1, 2018:

The Stoll group has completed development of a method involving large volume injections of aqueous samples (~ 1 mL), two-dimensional liquid chromatography separations, and detection by tandem mass spectrometry (QTOF). This method is sufficiently sensitive to detect photoproducts of tonalide and galaxolide at parts-per-trillion levels in water. However, the challenge that we have encountered recently is that it appears there is a tremendously complex mixture of interfering compounds present in river water that will make it very difficult to quantify the transformation products of tonalide and galaxolide with high confidence. This problem has not been reported in the existing literature. In the next months we will continue work to see if we can improve the specificity of the method. Two Gustavus undergraduate students (Amy Crawford and Devin Makey) were involved in this work in June and July – one supported by LCCMR funds, and one supported by internal research funds from Gustavus Adolphus College.

Activity Status as of February 1, 2019:

The Stoll group has worked on purifying large quantities of galaxolide for use in subsequent photodegradation studies. One Gustavus undergraduate student (Amy Crawford) has contributed to this work. Activity 2 is mainly focused on determination of musk parent compounds and their photoproducts in WWTP effluents. Our inability to obtain WWTP effluents has prevented us from completing these particular tasks. In lieu of these samples, we

have collected and analyzed samples from the St. Croix River downstream of WWTP discharge points. We did not detect any musks or their photoproducts in these samples (i.e., concentrations were below the detection limits of our methods). Also, while we have waited for samples, we have spent more time than initially expected working to understand the complexities of galaxolide that have not been previously reported on in the literature. This ranges from having to develop a robust purification method for the parent compound because it is not commercially available as a pure substance, to realization that the parent compound transforms rather quickly in completely aqueous solutions. This additional work, which was not originally planned as part of Activity 2, has been an indispensable precursor to reporting believable results from the analysis of WWTP effluent samples.

Final Report Summary:

In the final reporting period, the Stoll group has analyzed samples of WWTP effluent using the 2D-LC-MS/MS method developed in prior effort on the project. Samples were obtained from the wastewater treatment plants of Mankato, Cannon Falls, and Northfield, both prior to and after treatment of the effluent with UV light. At this point we can say with confidence that both the parent compound and some transformation products of tonalide have been detected in these samples at ppb levels. Given that the actual analyses of some of these samples occurred very late in the reporting period, data analysis is still ongoing to quantify exactly which transformation products were detected, and at what levels. We anticipate having concentrations quantified by mid-September.

The original work plan for Activity 2 was focused on the development of methods to detect transformation products of galaxolide and tonalide in WWTP effluents. The development of a 2D-LC-MS/MS method itself has gone well, and we have a method in place that enables direct analysis of 1-mL water samples with minimal sample preparation (i.e., only centrifugation to remove particulates), yet yields detection limits for the musk compounds in the parts-per-trillion range. However, progress toward use of this method for analysis of WWTP effluent samples has been impeded for two primary reasons (one of which led to an important discovery). First, several difficulties were encountered when working with galaxolide that were unanticipated, and to the best of our knowledge have not been reported in the literature. It became evident in our work that **pure galaxolide quickly transforms when exposed to water**. Since this does not seem to be appreciated by the research community, it calls all of the existing literature on the study of galaxolide in the environment into question, and we spent quite a lot of time working to understand the discrepancy between what we observe and what has been reported. Second, getting access to effluent samples from WWTP plants to analyze with the method we have developed was very challenging. Ultimately, we were able to analyze samples from three plants, however the samples were obtained at the very end of the project period, which is why we are still working on the analysis of data from this work.

V. DISSEMINATION:

Description: The results of this study will be disseminated through oral and poster presentations by the students and faculty involved in the project, briefings to the LCCMR as requested, and peer-reviewed publication. We also intend to present progress on this project periodically to relevant personnel working on related ENRTF projects who have been made aware of this project and may be interested in the results.

Status as of February 1, 2017: Brady Anderson, UST undergraduate student, will present a poster titled "Environmental photochemistry of the polycyclic musks tonalide and galaxolide" at the American Chemical Society Spring National Meeting in San Francisco, April 2-6, 2017.

Status as of August 1, 2017: The Martinovic lab established contact and exchanged knowledge with the MN Zoo staff working on another mussel ENTRF-sponsored project. The existing knowledge that the MN Zoo group has (mussel culture) is very helpful to our group and we agreed to keep in touch about the research plans to leverage each other's work. Furthermore, Martinovic-Weigelt traveled to Croatia for another project, but used

the opportunity to disseminate information about the ENTRF project to German and Croatian groups who are experts in this area. We plan to keep exchanging knowledge and methods with these groups to ensure that we are efficient and current (in regards to mussel-specific in vitro methods). Finally, in June 2017 Martinovic-Weigelt met with the MN Pollution Control staff, and participated in the MN Department of Health's 2017 Emerging Contaminants Stakeholder Forum where she had an opportunity to introduce the project to several participants from MDH and MPCA.

Status as of February 1, 2018: Carlee Heiling, UST undergraduate student, will present a poster titled "Photolysis and biological activity of the polycyclic musks tonalide and galaxolide" at the American Chemical Society Spring National Meeting in New Orleans, March 18-22, 2018. Martinovic-Weigelt established a collaboration with researchers at the Minnesota DNR (Lake City) and the Minnesota Zoo that will both advance the aims of this project and also lead to method development to facilitate mussel health monitoring and research by partner organizations.

Status as of August 1, 2018: Emily Stickney, UST undergraduate student, and Amy Crawford, GAC undergraduate student, are preparing abstracts to submit for presentation at the American Chemical Society Spring National meeting in Orlando, March 31 – April 4, 2019.

Status as of February 1, 2019: The Martinović-Weigelt lab presented research generated as a part of this project in November 2018 at the National Meeting of the Society of Environmental Toxicology and Chemistry in Sacramento, CA (circa 3,000 attendees; Teodor Grieder and Sarah Shadle were undergraduate student co-authors). UST student Sarah Shadle was also selected to present about the effectiveness of UV treatment for reduction of musk toxicity at the "Scholars at the Capitol" event on January 23 2019 at the MN State Capitol.

Final Report Summary: The Stoll and Wammer labs presented research results from this project in March 2019 at the National Meeting of the American Chemical Society (Orlando, FL; Amy Crawford (Stoll group) and Emily Stickney (Wammer group)), and the Stoll group also presented in June 2019 at the International Symposium on Liquid Phase Separations (Milan, Italy; Devin Makey).

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 92,986	Project manager 1 month of salary first two years, 0.5 month third year ($20,866); project partner 1 month of salary each year ($28,003); two undergraduate students full time in summer and an average of 7.5 hours per week during the academic year ($44,117).
Professional/Technical/Service Contracts:	$ 120,000	1 contract with Gustavus Adolphus College for assistance with identification of products and measurements of concentrations in WWTP effluents ($87,000); 1 contract with University of Minnesota Crookston for method development related to molecular/physiological endpoints for fish and mussel studies ($33,000).
Equipment/Tools/Supplies:	$ 61,429	Sample prep supplies ($3,500), cells and supplies ($4,400), general toxicity tests ($27,000), supplies for in vivo assays ($6,500), supplies for mussel assays ($4,600), general supplies for biological studies ($2,800), fish and mussels and supplies ($2,500), photolysis and

		chromatography supplies ($9,769), shipping costs ($360).
Capital Expenditures over $5,000:	$ 11,085	1 LuzChem UV photoreactor instrument.
Travel Expenses in MN:	$ 1,500	Mileage for obtaining WWTP effluent samples.
TOTAL ENRTF BUDGET:	**$ 287,000**	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: One LuzChem UV photoreactor is being purchased and will continue to be used by the University of St. Thomas for the life of the instrument for similar projects and purposes. 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.

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 2.5

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 2

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
In-Kind Support	$ 71,200	$ 71,200	Indirect costs (waived)
TOTAL OTHER FUNDS:	**$ 71,200**	**$**	

VII. PROJECT STRATEGY:

A. Project Partners:

Project partners receiving funds:

- Kristine Wammer and Dalma Martinovic-Weigelt, University of St. Thomas: $167,000 to measure UV photolysis rates, generate product mixtures for activity assay testing, isolate suspected active products (Wammer) and to lead work on toxicity assays and fish and mussel studies (Martinovic-Weigelt) (Activity 1).
- Anthony Schroeder, University of Minnesota – Crookston: $33,000 to develop molecular/physiological endpoints for fish and mussel studies (Activity 1).
- Dwight Stoll, Gustavus Adolphus College: $87,000 to assist with identification of products (Activity 1) and measure concentrations in WWTP effluents (Activity 2).

All project partners will supervise students.

B. Project Impact and Long-term Strategy: In addition to disseminating our work through peer-reviewed scientific publications and presentations, we will communicate and work with the PI of an existing ENRTF mussel study and MPCA personnel involved in WWTP effluent survey work as appropriate during the project. If warranted by our findings, we will collaborate with WWTPs statewide to introduce appropriate UV technologies that will facilitate removal of the toxic contaminants and be protective of fish and mussel health.

C. Funding History: N/A

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

A. Parcel List: N/A

B. Acquisition/Restoration Information: N/A

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

X. RESEARCH ADDENDUM: See attached Research Addendum.

XI. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted no later than February 1, 2017; August 1, 2017; February 1, 2018; August 1, 2018; and February 1, 2019. A final report and associated products will be submitted between June 30 and August 15, 2019.

Environment and Natural Resources Trust Fund
M.L. 2016 Project Budget

Project Title: Assessing Techniques for Eliminating Contaminants to Protect Native Fish and Mussels
Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Sub. 04d
Project Manager: Kristine H. Wammer
Organization: University of St. Thomas
M.L. 2016 ENRTF Appropriation: $287,000
Project Length and Completion Date: 3 Years, June 30, 2019
Date of Report: August 9, 2019 (final budget as of 6/30/19)

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Amount Spent	Activity 2 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	**Quantify removal of contaminants by UV treatment and measure toxicity and endocrine disrupting activity**			**Quantify contaminants and their UV products in municipal wastewater**				
Personnel (Wages and Benefits)	$92,986	$92,982	$4				$92,986	$4
Kris Wammer, Project Manager: 1 month of salary first 2 years, 0.5 month third year (432 hours total, .2 FTE total). Supervise students, coordinate project, compile and file reports, disseminate results. 93% salary, 7% fringe ($20,866).								
Dalma Martinovc-Weigelt, Project Partner: 1 month of salary each year (516 hours total, 0.25 FTE total). Supervise students, assist with coordinating project, disseminating results. 93% salary, 7% fringe ($28,003).								
Undergraduate students. 2 students during the academic year (one 5 hours per week for 32 weeks, one 10 hours per week for 32 weeks), 2 students each summer 40 hours per week for 11 weeks (4080 hours total, 2.0 FTE total). 95% salary, 5% fringe benefits ($44,117).								
Professional/Technical/Service Contracts								
Gustavus Adolphus College				$87,000	$72,974	$14,026	$87,000	$14,026
Dwight Stoll, Project Partner. 0.5 month of salary per year for first two years, 0.33 month salary for third year (229 hours total, 0.1 FTE total). Supervise Gustavus undergraduate students and reseach technician. 86% salary, 14% fringe benefits ($10,084).								

Research technician 10 hours per week in years 1 and 2, 7 hours per week in year 3 (1404 hours total, 0.7 FTE total). 75% salary, 25% fringe benefits ($42,100).								
Undergraduate students. 1 student during the first two summers (40 hours per week for 10 weeks) (80% salary, 12% housing, 8% fringe benefits). 1 student during the academic year, 8 hours per week for 15 weeks per semester, (100% salary) (1520 hours total, 0.7 FTE total). ($15,283)								
General lab supplies, e.g. solvents, vials, analytical standards ($7,483).								
LC/MS instrument access ($11,000).								
Travel (meetings with other groups, some sampling) ($1,050).								
University of Minnesota – Crookston	$33,000	$33,000	$0				$33,000	$0
Anthony Schroeder, Project Partner. 0.5 month of salary per year for first two years, 1 month of salary for third year (344 hours total, 0.2 FTE total). Supervise undergraduate student. 100% salary ($13,333).								
Undergraduate students. One student during the first and second academic years, 8 hours per week for 16 weeks per semester. One student during the first summer, 40 hours per week for 10 weeks. (912 hours total, 0.4 student FTE total). ($9476)								
Lab supplies, e.g. disposable plastics, primers, Sybr green mastermix, RNA extraction kits, enzyme assays, hormone assays ($10,091).								
Equipment/Tools/Supplies	$61,429	$61,426	$3				$61,429	$3
Sample filtration, extraction and preparation for all analyses - 20 samples @ $175/sample ($3,500).								
Cells/supplies, media, standards for endocrine in vitro asessments - 20 samples @ $220/sample ($4,400).								
90 general toxicity tests - parent/UV degradation compounds - 10 samples/ $30/sample/test ($27,000)								
Reagent supplies for in vivo molecular/physiological assessment (enzyme/hormone/ gene assays) - 45 ($6,500).								
Reagents and disposables for mussel MXR defense assays and nutrient chemistry ($4,600).								

Miscellaneous lab supplies (pipette tips, culture plates, tubing, sterile syringes/containers, assay plates ($2,800).								
Fish and mussels, holding supplies, and food ($2,500).								
General photolysis and chromatography supplies (e.g. columns, quartz tubes, reagents, solvents) ($9,769).								
Shipping costs to send samples between institutions for analysis ($360).								
Capital Expenditures Over $5,000	$11,085	$10,910	$175				$11,085	$175
LuzChem UV photoreactor instrument								
Travel expenses in Minnesota	$1,500	$150	$1,350				$1,500	$1,350
Mileage for obtaining WWTP effluent samples (6 treatment plants, at least twice per year; most sites in MN River Basin or St. Croix River)								
COLUMN TOTAL	**$200,000**	**$198,467**	**$1,533**	**$87,000**	**$72,974**	**$14,026**	**$287,000**	**$15,559**