Environment and Natural Resources Trust Fund

Research Addendum for Peer Review

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1. Abstract

Wastewater treatment plants discharge effluent that contains contaminants of emerging concern (CECs), including estrogens. These estrogens have caused dramatic ecological effects such as fish feminization and fish population collapses, with unknown long-term consequences. The most important estrogen exiting wastewater treatment plants, in terms of contributing to the feminization potential of effluent, is a chemical called estrone, which is an estrogen that is released naturally from women via the waste stream. Although this and other estrogens are present in Minnesota lakes and rivers and can be ecologically harmful, their treatment and discharge are not regulated.
Interestingly, the discharge of estrone and other estrogens is a function of how (and how well) a treatment plant removes nitrogen. Nitrogen discharge is regulated to some extent in Minnesota and will be more heavily regulated in the future, requiring additional wastewater treatment plant upgrades. With this research we will determine how different nitrogen removal processes perform over the range of temperatures experienced in Minnesota with respect to both CEC (and in particular, estrone) and nitrogen removal so that the very best processes for the protection of Minnesota's natural resources can be put into place. In addition, we will determine how fish vulnerability changes seasonally so that treatment to extremely low levels of CECs is only required during critical periods (e.g., during egg maturation or spawning) to save energy and costs from excessive (and unnecessarily rigorous) treatment. Finally, we will combine laboratory efforts with predictive mathematical models so that we can extrapolate to cost and whole population behavior.

2. Background

Wastewater treatment plants discharge effluent that contains nutrients such as ammonia (NH_3) and nitrate (NO_3^-) and contaminants of emerging concern (CECs) such as estrogens. NH_3 is toxic to aquatic wildlife and exerts an oxygen demand in the receiving water. NO_3^- is known to cause eutrophication and subsequent harmful algal blooms in the water bodies surrounding the state (Lake Superior, the Mississippi River, and Lake Winnipeg) ***(MPCA, 2013)***. Estrogens have caused dramatic ecological effects in fish such as behavioral ***(Schoenfuss et al., 2008; McGee et al., 2009)*** and physiological changes (e.g., feminization) ***(Routledge et al., 1998; Balch et al., 2004)*** and population collapses ***(Kidd et al., 2007)***, with unknown long-term and regional consequences. The most important estrogen exiting wastewater treatment plants, in terms of contributing to the feminization potential of effluent, is a chemical called estrone, which is an estrogen that is released naturally from women via the waste stream ***(Onda et al., 2003; Salste et al., 2007)***.

Wastewater treatment

In Minnesota, NH_3 discharge from wastewater treatment plants is regulated. NO_3^- discharge from wastewater treatment plants is regulated in the Lake Superior watershed but not elsewhere in the state. This will likely change, with the current draft Minnesota Nutrient Reduction Strategy calling for a 20% reduction in (total) wastewater nitrogen discharges by 2025 ***(MPCA, 2013)***. As a result, wastewater treatment plants throughout the state will be tasked with upgrading their nutrient removal technology, implementing either proven but energy-intensive biological nutrient removal (BNR) technology or investing in slightly more experimental, and lower energy use, approaches to remove total nitrogen.

The most basic BNR treatment plant configuration (the Modified Ludzack-Ettinger (MLE) process) typically consists of two zones: (1) an initial anaerobic zone followed by (2) an aerobic zone ***(Kang et al., 2008, Figure 1)***. In the anaerobic zone, influent organic carbon is degraded, NH_3 is produced, and any NO_3^- present is degraded to N_2, which is released to the atmosphere. In the aerobic stage, NH_3 in the wastewater is degraded to NO_3^-. The key to the removal of total nitrogen (i.e., NO_3^- degradation), is the recycle of a portion of the effluent from the second aerobic stage back to the initial anaerobic stage. This recycling causes the NO_3^- that was formed under aerobic conditions to be degraded to N_2 under the anaerobic, carbon-rich conditions found in the first stage. Though effective, the MLE process requires a great deal of operational control and is energy-intensive as a result of the second (typically longer) aerated step. One real benefit to systems like the MLE process, in terms of safeguarding ecological health, is the fact that the extended aeration steps in which NH_3 is transformed to NO_3^- have been shown to remove CECs such as estrogens ***(Clara et al., 2005; Koh et al., 2009; Tan et al., 2013)***. Therefore, although energy-intensive, the quality of the effluent is very high in terms of a lack of total nitrogen and other harmful compounds like estrone.

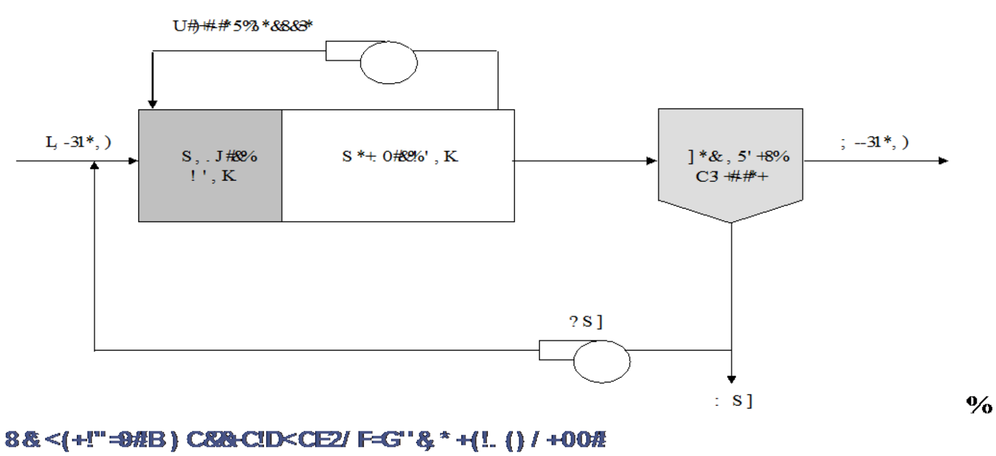

Figure 1. Taken from Kang et al. (2008). WAS is waste activated sludge. RAS is recycle activated sludge.

Multiple modifications of the basic MLE process exist ***(Kang et al., 2008)***, as well as newer, potentially lower-energy, technologies such as the ANAMMOX process ***(Kang et al., 2008)*** and low-oxygen granular sludge systems in which simultaneous NH_3 and NO_3^- degradation can occur at as a result of the enrichment of a unique granular biomass. Though more experimental, full-scale systems exist that make use of these newer technologies. Nevertheless, other side benefits, such as the ability of such systems to degrade harmful CECs such as estrone, have not been tested with these BNR systems.

Wastewater effects on fish

Treated wastewater effluent has been recognized as one of the primary sources of CECs to aquatic environments ***(Routledge et al., 1998; Kolpin et al., 2002)***. The presence of CECs in aquatic environments has been a growing concern due to evidence indicating the disruption of normal reproductive function in aquatic organisms ***(Norris and Carr, 2006; Norris, 2007)***. Vertebrate animals, including fishes, have an endocrine system consisting of glands that secrete hormones, such as 17β-estradiol, which maintain organismal homeostasis by regulating developmental and reproductive functions ***(Norris and Carr, 2005)***. Environmental estrogens that mimic 17β-estradiol have the ability to bind and activate estrogen receptors, thereby mimicking a normal endocrine response ***(Jobling and Tyler, 2003)***.

Environmental estrogens can cause changes to organismal homeostasis ***(Norris, 2007)*** and reproductive impairment ***(Miller et al., 2007)***. Numerous studies have reported adverse effects when fish were exposed to steroidal estrogens including increased plasma vitellogenin (an egg yolk precursor normally found in females, ***Panter et al., 2008***) concentrations in male fish, impaired spermatogenesis ***(Jobling et al., 2002***), disrupted gonadal morphology ***(Hinck et al., 2009; Purdom et al., 1994; Harries et al., 1997; Jobling et al., 1998)***, altered behavioral responses ***(Murphy et al., 2001; Belanger et al., 2007; Schoenfuss et al., 2008; McGee et al., 2009)*** and decreased reproductive success ***(Panter et al., 2008; Bjerselius et al., 2001; Parrott and Blunt 2005; Thorpe et al., 2009; Paulos et al., 2010)***. Most dramatically, the exposure of an entire lake in Canada for three summers to the synthetic estrogen ethinylestradiol resulted in the collapse of the resident fathead minnow (*Pimephales promelas*) population ***(Kidd et al., 2007)*** followed by a collapse of apex predator species as a result of the diminished prey source ***(Palace et al., 2009)***. Population modeling approaches have also indicated the potential of CECs to reduce population sustainability ***(Miller et al., 2007)***. The continuous release of these compounds through wastewater treatment plant discharge results in “pseudo persistent” scenarios where chemical half-lives are exceeded by effluent introduction rates (***Daughton, 2002)***.

Assessing the impact of wastewater CECs on individual fish and fish populations is complicated by the effects of temperature on CEC biodegradation during wastewater treatment and uptake of CECs by fish at various time points in their natural history. Concentrations of CECs are frequently highest during the spring when effluent temperatures are low ***(Barber et al., 2011; Lozano et al., 2012; Minarik et al., in press)***. In the Upper Midwest, effluent temperatures are usually 7-10°C colder during the winter and spring than in late summer and fall. Metabolic processes are generally governed by the Q10 rule, which predicts that microbial metabolic activity will double to triple if the temperature increases by 10°C ***(Banik and Daguet, 1997; White et al., 2006)***. Thus, the seasonal differences in effluent temperature in the Midwest may indicate that microbial degradation processes could be operating much slower during winter and spring, thus resulting in less CEC removal during this period. This potential seasonal increase in effluent CEC concentrations overlaps with a critical period in sexual maturation, gametogenesis and spawning in smallmouth bass (*Micropterus dolomieu*) (a predator species) and gametogenesis in fathead minnows (a prey species), suggesting higher exposure rates at this life stage (Panter et al., 2006). In contrast, larval development and organogenesis occurs later in spring (smallmouth bass) and summer (fathead minnow) when CEC concentrations are lower and water temperatures are higher (Devlin and Nagaharna, 2002). Nevertheless, the increase in water temperature may also imperil larval and juvenile fishes during the critical stages of organogenesis and sexual differentiation by elevating rates of metabolism and respiration (i.e., CEC uptake) (Devlin and Nagaharna, 2002). Clearly, decoupling temperature, CEC concentrations and their effects on different fish life stages is crucial in assessing critical periods in which enhanced wastewater treatment is needed.

Valuation of natural resources and water quality trading

Given a policy to reduce pollution, it is smart to have a system to meet a pollution standard that minimizes the cost of compliance. A simple rule that dictates that all polluters must reduce their emissions by a particular percentage is unlikely to be the cost-minimizing solution because some polluters can reduce their emissions with lower costs than others. This is particularly true for wastewater treatment plants, where some small towns would require expensive upgrades to facilities for small reductions in the mass of pollution discharged. Water-quality trading systems, properly designed, show promise to reduce pollution levels while minimizing the cost of compliance even without information about the cost of reducing pollution at the individual plant level.

The State of Minnesota, in its phosphorus pollution-trading program for the lower Minnesota River, has one of the few water pollution trading systems with a history of multiple trades. The abundance of trades speaks to the success of the program because trades occur when opportunities for cost reduction exist. Nationally, the Environmental Protection Agency and the United States Department of Agriculture have announced plans to encourage water quality trading across the country as a way to cost-effectively comply with Clean Water Act requirements.

Economists have identified two general designs for water quality trading. In one ***(Farrow et al., 2005)***, the trading unit is the amount of damage caused by a unit of pollution, denominated in dollars and calculated over the entire downstream waterway. In another ***(Hung and Shaw, 2005)***, the focus is on the amount of pollution in each zone in the river. The Minnesota example uses the “Jordan Trading Unit” (JTU), a measure of the contribution of the polluter to phosphorus concentrations in the Minnesota River as it flows through Jordan. Though the concept of water quality trading is relatively new, it offers an exciting way of looking at state-wide nutrient removal while incorporating other side benefits, such as the ability of wastewater systems to degrade harmful CECs such as estrone, to preserve additional natural resources such as fisheries.

<u>Summary and research needs</u>

The state of Minnesota will be requiring total nitrogen removal upgrades at wastewater treatment plants. Treatment plants are not only interested in removing total nitrogen, but are also interested in minimizing energy use and cost, which may lead to the exploration of some newer, lower-energy technologies such as ANAMMOX or granular low-oxygen systems. With the proposed research, we have an opportunity to address gaps in our knowledge of CEC removal and effects, while also being proactive regarding the need to remove nitrogen during wastewater treatment. To accomplish these goals, it is important to study the performance of treatment processes designed to remove total nitrogen over the range of temperatures experienced in Minnesota with respect to <u>both</u> CEC and nitrogen removal. In addition, we propose to determine how fish vulnerability changes seasonally so that treatment to harmless levels of CECs is only required during critical periods (e.g., during larval development or spawning) to save energy and costs from excessive (and unnecessarily rigorous) treatment. Finally, we propose combining laboratory efforts with predictive simulation models so that we can extrapolate to cost and whole population behavior. This approach will inform utility and regulatory personnel with respect to those nitrogen removal technologies that also offer more comprehensive protection of Minnesota's natural resources in terms of energy use and CEC degradation.

3. Hypotheses

Activity 1: Determine the performance of different wastewater treatment processes with respect to nitrogen removal, CEC and estrone removal, energy use, and cost**.**

Hypothesis: Systems that enrich for multiple substrate utilizers and have a long cell residence time, such as low-oxygen granular sludge systems and attached growth systems, will perform well in terms of total nitrogen, CEC, and estrone removal. The energy use of such systems will be lower, and thus the expected operational costs are lower. ANAMMOX is not expected to remove CECs and estrone well, despite the long residence time, because of a lack of aeration ***(Kvanli et al., 2008)***. A conventional MLE system will remove total nitrogen, CECs, and estrone effectively, but will have a higher operational cost associated with it.

Activity 2: Determine how temperature and life stage alter the reproduction and survival of fathead minnows and smallmouth bass after exposure to a series of estrone concentrations modeling the estrogenicity of real and treated synthetic wastewater effluent.

Hypothesis: The effects of estrogen exposure on individual fish will be modulated by water temperature and exposed life stage. Increased temperatures at any life stage will result in higher respiration rates, greater metabolic activity and hence will result in greater uptake of estrogens by the exposed organism. The permeability of eggs and large surface to volume ratio in larvae will result in greatest sensitivity of this life stage. The interactions between male and female fish during reproduction and the need for synchronized spawning will result in significant sensitivity of this life stage as well. Estrogen exposure will hinder the ability of fish to avoid predators. The extent to which higher temperatures exacerbate the effects of estrogen exposure is expected to differ between the biological responses introduced above.

Activity 3: Conduct an empirical analysis of alternative water quality trading systems and solve for the cost of attaining a set of water quality levels. Simulation modeling will link these water quality levels to the population biomass of fathead minnow and smallmouth bass.

Hypothesis: The desirability of a specific water quality trading system design depends upon the setting in which it will be implemented and the nature of damages caused by water pollution. Coggins et al. ***(2013)*** explains that nonlinearity in damages and the location of damages affect whether a particular system is appropriate. In addition, the linking of multiple pollutants (e.g., nitrogen and estrones) may suggest a trading system that incorporates both of these pollutants. In addition, the sensitivity of fish populations during specific seasons of the year suggests that the overall standard should vary with the calendar. We hypothesize that these factors will influence the cost of meeting water quality standards and that these costs will vary with the system design.

4. Methodology

Activity 1.

Five laboratory-scale reactor systems will be set-up to mimic different wastewater treatment systems, including conventional (NH_3 removal-only) treatment (*CONV*) and four treatment systems designed for total nitrogen removal. Reactors will be constructed from glass and will be designed to mimic the most basic BNR process (*MLE*, described above), a nitritation-denitritation system (*N-D*), *ANAMMOX*, and a low-oxygen granular sludge process (*GRAN*). The *N-D* process is similar to the *MLE* process except that the aerobic zone is smaller with a shorter residence time and is followed in series by a second anaerobic zone in which an external carbon source (methanol for example) may be added. The *ANAMMOX* process is similar to the *N-D* process except that the aerobic zone may be even smaller/shorter and no carbon is added to a much longer anaerobic process in which specialized bacteria, anammox bacteria, are seeded and cultivated. Anammox bacteria require warmer temperatures, a very long cell residence time, and a narrow pH range for successful operation. Finally, the *GRAN* process requires a feast-famine style of operation in which a high concentration of influent is fed to a vigorously mixed system to encourage granulation. All of the reactors, except for the *GRAN* reactor, will be fed continuously and operated as described in **Table 1** below. A membrane separation system will be used with each reactor set-up to retain the biomass for recirculation ***(Tan et al., 2013)***; this will allow more precise control of solids residence time, a critical parameter in wastewater treatment plant operation. Although initial sorption of estrone to the polycarbonate and the membrane is expected, this should reach a steady state after approximately 20 days ***(Tan et al., 2013)***. We anticipate being able to operate 2 reactor systems at a time, with the *ANAMMOX* system operated in isolation as a result of the more complex set-up required **(Table 1)**.

Table 1. Reactor operating conditions

Process	**Reactor configuration**	**Dissolved oxygen set-point**	**Solids residence time**	**Hydraulic residence time**
CONV	One reactor	5 mg/L	10 days	5 hours
MLE	Two reactors (A and B) in series	A: 0 mg/L B: 5 mg/L	Overall: 10 days	10 hours
N-D	Three reactors (A, B, and C) in series	A: 0 mg/L B: 5 mg/L C: 0 mg/L	Overall: 10 days	10 hours
ANAMMOX	Three reactors (A, B, and C) in series	A: 0 mg/L B: 5 mg/L C: 0 mg/L	A and B: 10 days C: 25 days	10 hours
GRAN	One sequencing batch reactor (to better model plug flow)	1-2 mg/L	Overall: 20 days	10 hours

Experiments will initially be performed at approximately 72°F followed by a second set of experiments performed at approximately 59°F. Two exceptions are the experiments performed with the *N-D* and *ANAMMOX* reactors, which require heating of the aerobic (both, 90°F) and second anaerobic (*ANAMMOX* only, 97°F) reactors to function optimally. In these cases the feed to the reactors will be heated. The energy used for heating will be incorporated into the cost calculations. When appropriate (see **Table 1**), reactors will be aerated. The flow rate of oxygen required to meet the dissolved oxygen set-point will be monitored daily and will be incorporated into energy utilization and cost calculations. Reactors will be fed 200 mg/L soluble chemical oxygen demand (COD)-containing synthetic wastewater (peptone, sodium acetate, dry meat extract, glycerol, potato starch, and skim milk powder) amended with 10 μg/L estrone ***(Tan et al., 2013)***. Reactors will be operated for three solids residence times prior to the collection of data to ensure steady state and data will be collected over an additional 2-3 solids residence times. Reactor effluents will be monitored for soluble COD, estrone, NH_3, NO_3^-, NO_2^-, and dissolved oxygen. Two of the experiments will be repeated in triplicate to verify reproducibility.

Once the reactors have been operated and the effluent data has been collected with the synthetic wastewater, two to three experiments will be repeated with influent secondary wastewater from the Metropolitan Plant in St. Paul, MN. This plant is large (approximately 180 MGD) and is dominated by domestic, rather than industrial, waste. In addition, previous research in our laboratory showed that the estrogenicity of the secondary influent of this plant was relatively stable over a two-year, multi-season period (25±14 ng/L estradiol equivalents in the secondary influent, n = 7). Our prior research showed secondary influent estrone concentrations at the Metropolitan Wastewater Treatment Plant of 77±52 ng/L; therefore, to enable comparison with the synthetic wastewater experiments, estrone (10 μg/L) will be added to the wastewater influent. Once at steady state, effluent soluble COD, NH_3, NO_3^-, NO_2^-, estrone, and dissolved oxygen will be monitored to determine how the presence of a complex feed containing a broad range of CECs impacts the degradation of estrone. The effluent will also be collected for 10 days, and the CECs present will be extracted via the use of solid phase extraction and HLB cartridges. This extract can be concentrated and amended to aquaria in egg and larval exposure experiments (described under Activity 2). This will provide additional exposure data with real wastewater; thereby providing confirmation of previously observed survival and reproductive trends (see below). This is similar to the procedure used in Kelly et al. ***(2014)***; nevertheless, if problems with effluent extraction occur, the exposure experiments will be set-up in close proximity to the reactors and effluent will be fed to them directly. We have experience operating similar systems ***(Tan et al., 2013)*** and anticipate that all of the experiments will be completed in approximately 2 years.

Activity 2.
We will employ a staggered blocked design using two life stages, multiple temperatures and five exposure treatments (**Table 2**: four estrone concentrations representing the range of estrogenicity measured in the various effluent treatments, well water control) conducted for two species to determine windows of vulnerability to estrogenic exposure. The life history of non-migrating North American fishes usually contains two life stages during which the fish are assumed to be particularly vulnerable to the effects of environmental estrogens: (i) the embryonic/early larval stage during which organogenesis occurs and (ii) the period during which adult fish produce gametes and reproduce ***(Colborn et al., 1993; Guilette et al., 1995; Gaikowski et al., 1996; Gray et al., 1999)***. We will expose two species of native freshwater fish (fathead minnow, smallmouth bass) to four estrone concentrations during both stages. These species were chosen because they are native to North America, widespread and abundant in many aquatic environments, readily available from controlled culture facilities, represent two levels of the aquatic food chain (fathead minnow – primary consumer; smallmouth bass – apex predator whose prey include juvenile fathead minnows), and have been used as model species for laboratory and field studies of CECs in the past ***(Hinck et al., 2009)***. Furthermore, the fathead minnow was designated a tier 1 screen organism for the effects of CECs ***(Ankley et al., 1998)*** and the bass represents a recommended test species in the establishment of water quality criteria ***(Stephen et al., 1985)***. Finally, the differing life history of these two species allows us to compare across the range of life histories in common North American fishes. This will allow us to compare the severity of effects on each life stage in each species and assess mode-of-action pathways. Similar biomarkers will be assessed in both species to allow for comparison of exposure effects and to reflect differing levels of organismal organization (see **Table 2** below). Both life stages and species will be exposed at two temperatures (59°F = 15°C; 72°F = 23°C) to mimic conditions across the life history stages of these fishes and to match the treatment

conditions in Activity 1. In addition, larval fish will be exposed to two additional temperatures (18°C, 21°C) to generate a more robust temperature response curve. This will allow us to test the responses of larval fish to a greater, and realistic, range of environmental temperatures. Data resulting from this activity will form the foundation to build population models in Activity 3 and will identify water temperatures that exacerbate estrogenic effects in exposed fishes.

Table 2. Experimental design matrix for the proposed study (Activity 2). Both species of native freshwater fish will be exposed at two critical life stages to four concentrations of estrone that mimic estrogenicity ranges measured for real wastewater and experimentally treated wastewater (see Activity 1) or a well water control. Exposure length and endpoints to be analyzed are listed. The exposures will be conducted at four environmentally relevant water temperatures for larval fish (59°F = 15°C; 18°C; 21°C; 72°F = 23°C) and two temperatures for adult fish (15°C; 23°C).

	21-day exposure *Embryonic/ early larval stage*	21-day exposure *Adult stage*
Fathead minnow	• 5 days embryonic + 16 days post-hatch exposure • viability, hatching and survival of larvae • vitellogenin analysis • predator avoidance performance • prey capture performance	• 6 months post-hatch • vitellogenin analysis • fecundity and fertility • nest defense assay • histology • prey capture performance
Smallmouth bass	• 5 days embryonic + 16 days post-hatch exposure • viability, hatching and survival of larvae • vitellogenin analysis • predator avoidance performance • prey capture performance	• 18 months post-hatch • vitellogenin analysis • fecundity and fertility • nest defense assay • histology • prey capture performance

Exposures. All exposures will be conducted in the St. Cloud State University Aquatic Toxicology facility. This facility was designed specifically for use in CEC exposure experiments. The facility contains a dedicated in-house well that supplies temperature-controlled water via stainless steel plumbing to the exposure facility. All exposure standard operating procedures and quality assurance/quality control protocols are well established ***(Schoenfuss et al., 2008; McGee et al., 2009; Hyndmann et al., 2010; Shappell et al., 2010; Dammann et al., 2011; Schultz et al., 2011)***. Embryonic/larval exposures will utilize a 50% static renewal exposure system as in previous published studies ***(McGee et al., 2009; Schultz et al., 2011).*** Adult fish exposures will use a flow-through system as in previous published studies ***(Schoenfuss et al., 2008; Hyndmann et al., 2010; Dammann et al., 2011)*** to expose fish to four estrone concentrations (see Activity 1) or a well water control. An aquarium turn-over rate of four turn-overs/24 hr for the adult exposures will assure match the productivity of the flow-through treatment reactors and assure that chemical degradation and bioavailability of estrone is not a compounding factor in any of the exposure experiments. Fish of both species and life stages will be maintained at a constant 16:8 hr light:dark photoperiod and fed *ad libitum* a mixture of freshly hatched (larvae) or frozen (adult) brine shrimp augmented with frozen blood worms. This food source avoids any introduction of CECs via diet, as soy (a food source rich in phytoestrogens) is often used in the manufacture of flake food. All fish maintenance conditions will closely follow well-established US EPA protocols ***(Denny, 1987)***. Exposures will be conducted separately for the two life stages and species to reduce logistical complexities. However, our exposure design will be consistent across species and life stages and will only be adjusted when required for biological reasons (greater biomass of bass). To allow for sufficient processing time at the end of exposures, the starting day for each estrone concentration will be staggered by one day. Concentrations of CECs in the exposure systems will be measured as described in Activity 1 to assure that laboratory exposure experiments match environmental conditions. Reproductive assays with mature smallmouth bass will be conducted at an outdoor research facility with flow-through Mississippi River water to provide conditions needed to achieve reproduction in this species. However, the initial exposure of these fish will take place in the SCSU facilities.

Embryonic/ Early Larval Exposures. Fertilized eggs from breeding pairs of the two study species will be collected immediately following egg deposition on spawning tile (fathead minnow) or nest depression (smallmouth bass). Smallmouth bass eggs and adults will be obtained from a well-established fish hatchery program (10,000 Lakes Hatchery, Osakis, MN) and fathead minnow eggs and adults will be purchased from our long-time laboratory fish supplier Environmental Consulting & Testing (Superior, WI). Forty eggs (less than 12 hours post-deposition) per aquarium will be placed in small stainless steel mesh containers suspended inside the aerated exposure aquaria until hatching. The stainless steel mesh has large enough diameter gaps to allow hatched larvae to move out of the mesh and into the tank. Larvae will be provided twice daily with hatched A*rtemia* (brine shrimp) larvae although the larvae will largely rely on their yolk-sack for nutrients during the early larval exposure. At 16 days post-hatch, larvae will be collected to determine hatching success. Larvae will be assessed for predator avoidance and prey capture performance. The latter is an innate behavior easily disrupted by exposure to CECs ***(McGee et al., 2009; Painter et al., 2009; Schultz et al., 2012)*** and more recently shown to be of ecological relevance ***(Rearick, 2012).*** To avoid pseudo-replication, egg clutches from different breeding pairs will be treated as experimental units and assessed separately. After predator avoidance performance has been measured, larvae will be sacrificed, measured for length and weight, homogenized, and stored at -80°C for later use in determining vitellogenin gene expression. A set of validation experiments will be conducted near the end of the study to examine how closely the responses of fish to real/synthetic effluent treatments match the responses observed during estrogen exposure experiments. In these experiments, we will expose eggs and larvae to real and synthetic effluents. The collection of the effluents will be as described above in Activity 1, which we have used previously ***(Kelly et al., 2014)***. If problems are encountered with the extraction of effluent, experiments will be set up such that effluent can be directly fed to the exposure experiments. Procedures for exposure and the measurement of biological endpoints will be as described above. The resultant data will be used to develop the fish biomass model described in Activity 3.

Adult Exposures. Mature fish will be obtained from a fish culture facility (fathead minnows: Environmental Consulting & Testing, Superior, WI; smallmouth bass: 10,000 Lakes Hatchery, Osakis, MN). Both species will be exposed to the same treatment concurrently. Single sex groups of fish (4 replicates per sex/treatment for fathead minnows; six for smallmouth bass) will be exposed in 40-L exposure aquaria. Ten mature fathead minnows (n=40/treatment), or 2 mature bass per aquarium (n=12/treatment) will be exposed for 21 days to the range of estrone concentrations determined in Activity 1 to reflect the range of wastewater estrogenicities expected to be found in real wastewater under multiple treatment regimes. At the end of the exposure, we will assess prey capture success and reproductive behaviors of breeding pairs as described above and in previous studies ***(Dammann et al., 2011; Schultz et al., 2012)***. Female fish will be sacrificed, measured for length and weight, bled for plasma vitellogenin analysis, and dissected to remove livers (gene expression) and ovaries (fecundity; histopathology). Both species conduct paternal nest care; therefore, male fish will be placed into individual tanks and a nest defense assay ***(Hyndman et al., 2010; Dammann et al., 2011; Schultz et al., 2011, 2012)*** will be performed to assess whether synthetic or real treated wastewater effluent exposure reduced the androgen-driven nest defense behavior of these fish. After the behavior assay, males will be sacrificed, measured for length and weight, bled for plasma vitellogenin analysis, and dissected to remove livers (gene expression) and testis (gametogenesis; histopathology). The resultant data will be used to develop the fish biomass model described in Activity 3.

Activity 3.
During Activity 3 we will conduct an empirical analysis of alternative water quality trading systems and solve for the cost of attaining a set of water quality levels. Mathematical modeling will link these water quality levels to fathead minnow and smallmouth bass biomass. To link treatment options to fish biomass, we will develop a mathematical simulation model that uses environmental cues (e.g., seasonal temperature) and fish biology to predict minnow and bass biomass under various scenarios of exposure to treated effluent. This information will allow us to express the cost of treating effluent in terms of benefits related to the biomass of different fish species.

Mathematical Simulation Model. We will model the equilibrium biomass of fathead minnows and smallmouth bass in a river that receives treated wastewater. This river will resemble a large river in Minnesota (e.g., Minnesota, Mississippi, St. Croix) in terms of channel dimensions and seasonal flow rate; we can, however, tailor these parameters to any hydrologic system/regime of interest. CECs will enter this system on a daily time step via a wastewater outfall. We will model the concentration of CECs in this wastewater as a function of the temperature in the treatment plant (modeled after local observations) and treatment (Activity 1). We will model CEC concentration downstream of this input via a numerical flow and transport model (Delft3D) that accounts for downstream diffusion and dynamics (e.g., temperature-dependent decay, chlorination).

We will base the latter on known functions (e.g., Williams et al. 2009) as well as unpublished data collected by Dr. Schoenfuss and collaborators..

A spatially-explicit, individual-based population model will be used to estimate fathead minnow and smallmouth bass biomass, both in the absence of wastewater and under different scenarios of wastewater treatment. This model will include consumption, growth, mortality, movement, and reproduction. Fish will consume a generic prey base and, in the case of smallmouth bass, fathead minnows. Consumption and subsequent growth will be governed by a conventional bioenergetics approach ***(Hanson et al., 1997)***, dynamic energy budget theory ***(Kooijman, 2000)***, and CEC toxicity as a function of concentration and temperature (Activity 2). We will model mean daily river temperatures after local observations (e.g., in-stream records, weather station data). Fish in the model will experience starvation as well as a background rate of natural mortality that is modified by predation and CEC toxicity (Activity 2). Individuals will move according to a fitness algorithm that weighs expected mortality against expected weight change. Individuals that survive and mature will spawn eggs seasonally until senesce at a maximum age. Individual egg production will be determined by individual size and both physiological and behavioral effects of CEC toxicity (Activity 2). Eggs and larvae will develop according to temperature, and egg and larval survival will be modified by CEC toxicity (Activity 2).

Initially the long-term (i.e., years to decades) equilibrium biomass of both fathead minnows and smallmouth bass will be simulated in the absence of wastewater. This simulation will be calibrated such that the biomass densities of fathead minnows and smallmouth bass are typical of prey and predator fish in the type of system that is being modeled. We will then simulate the long-term, equilibrium biomass of both fathead minnows and smallmouth bass under different scenarios of wastewater treatment. The upstream and downstream distances at which fathead and smallmouth biomass are indistinguishable from the pristine (no wastewater input) case will define the spatial extent of these simulations. We will use the relative change in fathead minnow and smallmouth bass biomass (pristine vs. CEC-exposed) as a measure of the impact of a particular treatment scenario.

Cost-Benefit Analysis. What is the actual cost of attaining increasingly stringent pollution standards, and how do these costs depend on the system design? Zajicek ***(2013)*** produced preliminary estimates for the phosphorus trading system, and found large savings. Using information about costs of upgrading wastewater treatment facilities and ongoing operating costs from Activity 1 and from the literature, we will first estimate the costs of upgrading individual plants of different sizes. Jiang et al. (2004) document the economies of scale in wastewater treatment from detailed EPA data and estimate the costs of building plants employing alternative treatment technologies. We will build on their approach using the designs outlined in Activity 1. Then, we will estimate the overall system-wide costs of meeting water quality goals. These costs will depend upon the particular choice of water quality trading system, so models of water quality trading will be developed that mirror the different options. These will correspond, through the fishery model, with reduced damages to the population of fathead minnow and smallmouth bass.

5. Results and Deliverables

In this project we will determine 1) the total nitrogen, estrone, and CEC removal efficiency of 5 different wastewater treatment plant configurations under two different operating temperature regimes, 2) the effect of expected effluents on two species of fish (small mouth bass and fathead minnows) at two different life stages (corresponding to the different temperature regimes investigated), 3) the predicted minnow and bass abundance under various wastewater treatment scenarios, and 4) the cost-benefit of different wastewater treatment processes in terms of fish abundance and plant operating costs. This information should be able to be used directly by utility personnel, environmental consultants, and state agency personnel to move forward with specific recommendations for the best technologies to be implemented to achieve total nitrogen removal concomitant to CEC and estrone removal coupled with energy savings.

6. Timetable

This project is a three-year project, beginning in July, 2014. The timetable for completion of the described project follows in table format, divided into 3-month (quarter) increments.

	Quarter											
Tasks	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
CONV and *MLE* reactor construction	**X**											
CONV and *MLE* reactor operation to steady state (72°F)	**X**											
CONV and *MLE* reactor effluent analysis (72°F)	**X**											
CONV and *MLE* reactor operation to steady state (72°F) (replicate)		**X**										
CONV and *MLE* reactor effluent analysis (72°F) (replicate)		**X**										
CONV and *MLE* reactor operation to steady state (72°F) (second replicate)		**X**										
CONV and *MLE* reactor effluent analysis (72°F) (second replicate)			**X**									
CONV and *MLE* reactor operation to steady state (55°F)			**X**									
CONV and *MLE* reactor effluent analysis (55°F)				**X**								
N-D, *GRAN*, and *ANAMMOX*				**X**								

reactor construction												
N-D reactor operation to steady state (90°F)				**X**								
N-D reactor effluent analysis (90°F)				**X**								
ANAMMOX reactor operation to steady state (97°F)					**X**							
ANAMMOX reactor effluent analysis (97°F)						**X**						
GRAN reactor operation to steady state (72°F and 55°F)							**X**					
GRAN reactor effluent analysis (72°F and 55°F)							**X**					
Repeat two reactor runs with actual wastewater, chosen based on initial results (operation to steady state)								**X**				
Repeat two reactor runs with actual wastewater, chosen based on initial results (effluent analysis)								**X**				
Embryonic and larval fathead minnow and bass exposure experiments			**X**	**X**	**X**	**X**	**X**					
Mature fathead minnow and bass reproduction experiments					**X**	**X**	**X**	**X**	**X**			
Analysis of biological data						**X**	**X**	**X**	**X**	**X**		
Model development, parameterization, and calibration (pristine)					**X**	**X**	**X**					
Model parameterization and simulations (CEC-exposed)								**X**	**X**	**X**		
Cost-Benefit Analysis					**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Prepare manuscripts for the dissemination of results (oral dissemination at local and national conferences or meetings will occur throughout the project)											**X**	**X**

7. Budget

The revised budget is provided below. A budget justification follows.

BUDGET ITEM	AMOUNT
Paige Novak, PI ($12,700 salary, $4,300 fringe, 33.6% fringe rate; total for 2 years; 3.8% effort)	$17,000
Paul Venturelli, Co-PI ($4,300 salary, $850 fringe, 19.8% fringe rate; total for 2 years; 1.9% effort)	$5,150
Frances Homans, Co-PI ($5,650 salary, $1,400 fringe, 24.7% fringe rate; total for 2 years; 1.9% effort)	$7,050
One Postdoctoral Researcher ($82,400 salary, $17,100 fringe (includes healthcare); total for 2 years; performing the mathematical modeling of fish populations)	$99,500
Two Graduate Research Assistants ($84,700 salary, $69,400 fringe (includes healthcare and tuition); total for 2 years for each student; one student will perform the research on the removal of nitrogen and CECs during wastewater treatment and the other will perform research onthe cost and value of wastewater treatment upgrades with respect to the preservation of fish populations))	$154,100
Subcontract: Some of the work will be conducted at St. Cloud State University (Activity 2). The subcontract amount will include salary for a research technician/postdoc ($48,825 salary, $17,575 fringe (36% fringe rate) per year for 2 years) and supplies for experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000/3 years).	$186,800
Equipment/Tools/Supplies: Laboratory supplies and analytical costs (includes, but is not limited to, chemicals for all analyses, supplies to maintain analytical equipment, supplies for reactor construction, and pumps ($30,000/3 years)).	$30,000
Travel: Travel between St. Cloud and Minneapolis for research progress meetings (in state)	$400
TOTAL ENVIRONMENT AND NATURAL RESOURCES TRUST FUND $ REQUEST	$500,000

V. OTHER FUNDS

SOURCE OF FUNDS	AMOUNT
Other Non-State $ Being Applied to Project During Project Period: none.	$-
Other State $ Being Applied to Project During Project Period: none.	$-
In-kind Services During Project Period: Novak will provide unpaid time to the project (1% cost-share). Because the project is overhead-free, laboratory space, electricity, and other overhead costs are provided in kind. The University of Minnesota overhead rate is 52%.	$232,000 (estimated)
Remaining $ from Current ENRTF Appropriation (if applicable): no prior projects directly related to proposed project.	$-
Funding History: The PIs have been supported by various agencies to study estrogen biodegration in wastewater treatment plants and the impacts of estrogens on fish.	$- (cannot be estimated)

Budget justification
Personnel
Over the course of the 3-year project, two years of support for two graduate students (Activity 1 and the economic aspects of Activity 3) and two years of support for one postdoctoral researcher and one research technician/postdoctoral researcher (Activity 2 and the population modeling aspects of Activity 3) are budgeted. Funds for the research technician/postdoctoral researcher will be covered under a subcontract to St. Cloud State University (see below).

The PI (Novak) will each receive 2 weeks of salary a year for the first 2 years of the project. The Co-PIs Venturelli and Homans will each receive 1 week of salary a year for the first 2 years of the project. No salary is requested for Schoenfuss who will be granted one semester 100% re-assign time by St. Cloud State University to focus on the analysis of biological data. The PIs will be responsible for project oversight, guidance of the graduate students and postdoctoral researchers/research technician, data interpretation and analysis, and report preparation and submission. Two graduate student research assistants will each devote 100% of their research time to the project over a 2-year period. Fringe benefits for graduate students include tuition, health insurance, and summer FICA. All fringe benefit rates are set by the University of Minnesota and St. Cloud State University.

Materials and Supplies
Funds ($30,000) are requested for materials, supplies, consumables, analytical costs and upkeep associated with the LC-MS, computers (to be used only on this project), and software. Required materials include, but are not limited to: pipette tips, glassware, solid phase extraction cartridges for extractions, chemicals for standards and experiments, pumps, analytical consumables, analytical fees, solvents, reagents, fish, gloves, digital data storage media, and laboratory notebooks.

A portion of the Materials & supplies are budgeted for support of the fish exposure experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000/3 years) and will be part of the subcontract to St. Cloud State University (see below).

Travel
Travel funds are extremely minimal ($400) are included for travel to meetings at either St. Cloud State University or the University of Minnesota for project coordination.

Subcontract (to St. Cloud State University)
The subcontract amount ($186,800) will include salary for a research technician/postdoctoral researcher ($48,825 salary, $17,575 fringe (36% fringe rate) per year for 2 years) and supplies for experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000 for 3 years) to complete Activity 2.

Total amount proposed
The total proposed project amount is $500,000. No indirect costs for the University of Minnesota or St. Cloud State University are included in the budget.

8. Credentials

Paige J. Novak

Professor, Environmental Engineering, Department of Civil Engineering
B.S., Chemical Engineering, 1992, The University of Virginia, Charlottesville, VA.
M.S., Environmental Engineering, 1994, The University of Iowa, Iowa City, IA.
Ph.D., Environmental Engineering, 1997, The University of Iowa, Iowa City, IA.

Research

Research interests are in the areas of hazardous substance biodegradation, anaerobic biological processes, and the occurrence and fate of estrogenic compounds. Current research focuses on the enhanced transformation of chlorinated compounds in the presence of anaerobic organisms and the treatment of plant-based estrogens in industrial wastewater. Dr. Novak was the 2007 recipient of the Paul L. Busch Award (Water Environment Research Foundation) for her research on industrial phytoestrogens, the 2013 Bill Boyle Educator of the Year Award (Central States Water Environment Association), and the 2011 Samuel Arnold Greeley Award (American Society of Civil Engineers).

Selected Publications (51 total)

Tan, D. T., Arnold, W. A., Novak, P. J. 2013. Impact of Organic Carbon on the Biodegradation of Estrone in Mixed Culture Systems. *Environmental Science and Technology*, in press.

Rearick, D. C., Fleischhacker, N. T., Kelly, M. M., Arnold, W. A., Novak, P. J., Schoenfuss, H. L. 2014. Phytoestrogens in the Environment: I. Occurrence and Exposure Effects on Fathead Minnows. *Environmental Toxicology and Chemistry*, 33(3):553-559.

Kelly, M. M., Fleischhacker, N. T., Rearick, D. C., Arnold, W. A., Schoenfuss, H. L., Novak, P. J. 2014. Phytoestrogens in the Environment: II. Microbiological Degradation of Phytoestrogens and the Response of Fathead Minnows to Degradate Exposure. *Environmental Toxicology and Chemistry*, 33(3):560-566.

Lundgren, M. S., Novak, P. J. 2009. Quantification of Phytoestrogens in Industrial Waste Streams. *Environmental Toxicology and Chemistry*, 28:2318–2323.

Schnobrich, M. R., Chaplin, B. P., Semmens, M. J., Novak, P. J. 2007. Stimulating Hydrogenotrophic Denitrification in Simulated Groundwater Containing High Dissolved Oxygen and Nitrate Concentrations, *Water Research*, 41(9):1869-1876.

Heiko Schoenfuss

B.S., Biology, 1991, University of Bayreuth, Germany.
M.S., Veterinary Anatomy, 1997, Louisiana State University, Baton Rouge, LA.
Ph.D., Evolutionary Morphology, 1997, Louisiana State University, Baton Rouge, LA.

Research
Developing integrated analysis methodology to assess the effects of emerging contaminants on the aquatic life from the molecular level via organismal effects to trophic cascade consequences. Current research focuses on the effects of estrogenic endocrine active compounds and pharmaceuticals on the reproductive fitness of aquatic vertebrates.

Selected Peer-Reviewed Publications (66 total)
Minarik TA, Vick JA, Schultz MA, Bartell SE, Martinovic-Weigelt D, Rearick DC, Schoenfuss HL. In press. On-site exposure to treated wastewater effluent has subtle effects on male fathead minnows and pronounced effects on carp. *Journal of the American Water Resources Association.*

Martinovic-Weigelt D, Minarik TA, Curran EM, Marschuk JS, Pazderka MJ, Smith EA, Goldenstein RL, Miresse CL, Matlon TJ, Schultz MM, Schoenfuss HL. In press. Environmental estrogens in an urban aquatic ecosystem: I. spatial and temporal occurrence of estrogenic activity in effluent-dominated systems. *Environment International.*

Schultz MM, Minarik TA, Martinovic-Weigelt, Curran EA, Bartell SE, Schoenfuss HL. In press. Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. *Environment International.*

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L. *In Press*. Antidepressants at Environmentally Relevant Concentrations Affect Predator Avoidance Behavior of Larval Fathead Minnows *(Pimephales promelas)*. *Environmental Toxicology & Chemistry.*

McGee, M.R., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Schoenfuss, H.L. 2009. Predator Avoidance Performance of Larval Fathead Minnows (*Pimephales promelas)* Following Short-term Exposure to Estrogen Mixtures. *Aquatic Toxicology* 91: 355-361.

Schoenfuss, H.L., Levitt, J.T., Rai, R., Julius, M.L., and Martinovic, D. 2008. Treated wastewater effluent reduces sperm motility along an osmolality gradient. *Archives of Environmental Contamination and Toxicology*. DOI 10.1007/s00244-008-9219-1.

Paul Venturelli

Assistant Professor, Department of Fisheries, Wildlife and Conservation Biology, University of Minnesota
B.S., Environmental Science, 2000, York University
M.S., Biological Sciences, 2003, University of Alberta
Ph.D., Ecology and Evolutionary Biology, 2009, University of Toronto

Research

Dr. Venturelli is an expert in fish population dynamics and modeling. His research examines how temperature, habitat, life history (e.g., growth, maturity, reproduction, longevity) and human activities shape the population dynamics of fish species that are of interest to management and policy. He will direct the modeling effort to predict the effects of CEC exposure on fathead minnow and smallmouth bass biomass. He has been modeling fish populations for 7 years, including individual-based modeling for the past 3 years.

Selected Publications (9 total)

Lester NP, BJ Shuter, PA Venturelli, D Nadeau (in press) Life history plasticity and sustainable exploitation: a theory of compensation applied to regional management of walleye. Ecological Applications.

Venturelli PA, NP Lester, TR Marshall, BJ Shuter (2010) Consistent patterns of maturity and density-dependent growth among populations of walleye (Sander vitreus): application of the growing-degree-day metric. Canadian Journal of Fisheries and Aquatic Sciences 61: 1057-1067.

Venturelli PA, CA Murphy, BJ Shuter, TA Johnston, PJvC deGroot, PT Boag, JM Casselman, R Montgomerie, MD Wiegand, WC Leggett (2010) Maternal influences on population dynamics: evidence from an exploited freshwater fish. Ecology 91: 2003-2012.

Venturelli PA, BJ Shuter, CA Murphy (2009) Evidence for harvest-induced maternal influences on the reproductive rates of fish populations. Proceedings of the Royal Society of London, B 276: 919-924.

Venturelli PA, WM Tonn (2006) Diet and growth of northern pike in the absence of prey fishes: initial consequences for persisting in disturbance-prone lakes. Transactions of the American Fisheries Society 135: 1512-1522.

Frances Homans

Professor and Interim Department Head, Applied Economics, University of Minnesota
B.A., Religion, 1983, Pomona College
M.S., Agricultural Economics, 1991, University of California at Davis
Ph.D., Agricultural Economics, 1993, University of California at Davis

<u>Research</u>
Recent research has focused on the economics of invasive species management, including a project to develop decision-making tools that could be used to allocate scarce forest pest management resources. Dr. Homans' interests also include the economics of land preservation in urban environments and regulated open access resource use. In 2010, Dr. Homans won the Publication of Enduring Quality Award from the Association of Environmental and Resource economists.

<u>Selected Publications (24 total)</u>
Horie, T., Haight, R.G., Homans, F.R., Venette, R.V. 2013. Optimal strategies for the surveillance and control of forest pathogens: A case study with oak wilt, *Ecological Economics*, 47 (3) pp. 506-517.

Homans, F.R., Smith, D. 2013. Evaluating management options for aquatic invasive species: concepts and methods. *Biological Invasions*, 15 (1) pp. 7-16.

Homans, F.R., Horie, T. 2011. Optimal detection strategies for an established invasive pest. *Ecological Economics*, 70 (6) pp. 1129-1138.

Mathews, L.G., Homans, F.R., Easter, K.W. 2002. Estimating the benefits of phosphorus pollution reductions: An application in the Minnesota River. *Journal of the American Water Resources Association* 38(5)pp. 1217-1223.

Homans, F. R., Wilen, J.E. 1997. A model of regulated open access resource use. *Journal of Environmental Economics and Management*, 32 (1) pp.1-21.

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9. Dissemination and Use

The target audience for results from this research will be professionals in the areas of wastewater treatment and natural resource management. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the DNR and MPCA, and environmental consultants. Results will be disseminated through scholarly publications in peer-reviewed journals such as *Environmental Science and Technology*. Results from the research project will also be presented at regional conferences such as the *Minnesota Water* conference and if possible, at targeted seminars at the DNR and MPCA. Results will be used to determine which wastewater treatment upgrades offer the most ecological protection while incorporating the value of fisheries and energy use.

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