Environment and Natural Resources Trust Fund

Research Addendum for Peer Review

Project Manager Name: Paige J. Novak, Ph.D., P.E.
Project Manager Email address: novak010@umn.edu
Project Title: Ecological Impacts of Effluent in Surface Waters and Fish
Project number: 011-A2

1. Abstract

Phytoestrogens are plant-based compounds that mimic estrogen and can therefore interfere with normal biological development. We recently discovered that some plant-processing industries had high effluent concentrations of the phytoestrogens daidzein and genistein, with lower concentrations of coumestrol, formononetin, biochanin-A and the estrogenic mycotoxin zearelenone present in some of the effluents. The biological effects of these compounds have not been well-studied, although it is known that they can feminize male fish. In addition, almost nothing is known about their environmental fate. When these compounds enter rivers and streams, it is likely that they will be degraded and may have a lessened impact on biota as a result, but this needs to be confirmed. We plan to determine the persistence of genistein and daidzein by studying the reactions that transform these compounds in the environment (reactions with sunlight, naturally-occurring bacteria, and sediment) to predict their fate in natural waters. We will also perform exposure experiments at realistic environmental concentrations to determine the impact of genistein and daidzein on fathead minnows, an important component of the Minnesota aquatic food chain. Genistein and daidzein were selected for detailed study as 1) they are known to feminize fish although as mentioned above, little is known about this, 2) it is known that high levels of genistein and daidzein in particular are discharged from industrial facilities (up to 250,000 ng/L), and 3) it is known that high levels of genistein and daidzein are present in plants. This research will enable us to predict the concentrations of genistein and daidzein in natural waters and their expected ecological impact. Sampling of water downgradient of two plant-processing facilities, along with fish exposure studies in the sample locations will be used to determine the concentrations and effects of these compounds in the environment. This will facilitate continued economic development in the state in an environmentally sensitive manner.

2. Background

It is well-documented that a large variety of chemical compounds can function as hormone mimics when released into the environment, altering natural endocrine signaling in wildlife [1]. Phytoestrogens, natural plant-derived estrogen mimics, have the ability to disrupt the endocrine system as well [1]. Phytoestrogen exposure has been shown to affect reproductive processes in many different species ranging from mice [2] to fish [3]. In particular, effects on fish are of concern as phytoestrogens may

be discharged to surface waters. In fact, studies have shown that phytoestrogens can lead to vitellogenin gene expression [4], immunosuppression [5], and decreases in testosterone production [6] and the intensity of aggressive behavior [7] in fish.

Regretfully, no studies to date have been performed to determine the absolute aqueous concentration of phytoestrogens where one would expect to see negative physiological impacts on aquatic organisms. Thorpe et al. [8] investigated vitellogenin induction as a result of estradiol-17β (E2) exposure in juvenile rainbow trout. Median effective concentrations for E2 were between 19 and 26 ng/L. Latonnelle et al. [9] investigated the relative binding affinity of various estrogens, including E2, genistein, daidzein, coumestrol, formononetin and biochanin-A, to the estrogen receptor in rainbow trout. Genistein was found to bind 81.9 times less-readily than E2. One can therefore estimate that an aqueous genistein concentration of 1.56 µg/L to 2.14 µg/L could induce vitellogenin production in rainbow trout [8, 9]. Similar calculations can be made for daidzein (24.6 to 33.8 µg/L), coumestrol (1.08 to 1.49 µg/L), formononetin (0.71 to 0.97 µg/L), and biochanin-A (271 to 371 µg/L). Nevertheless, this is simply an estimate and there is also evidence that phytoestrogens have differing binding affinities for the estrogen receptors of different fish species. One study of trout and sturgeon estrogen receptors showed that the relative binding affinity for five phytoestrogens varied and the difference among the compounds was as great as a factor of 12 [9], yet in terms of vitellogenin production, sturgeon were 50 times more sensitive to genistein than trout [10]. Combination effects of estrogenic compounds are also well documented [11, 12] and indicate that different estrogenic compounds can work in an additive fashion when disrupting endocrine function. It is currently unknown what effect mixtures of phytoestrogens discharged to surface waters may have on fish living in that water.

Despite evidence of their estrogenic effects on aquatic organisms, little work had been performed on identifying point sources of phytoestrogen discharge to surface waters until recently. Indeed, the focus with respect to phytoestrogen discharge had been on the pulp and paper industry, which has long been identified as a phytoestrogen point source. Pulp and paper mills have been shown to have high levels of isoflavones and phytosterols in their final effluent, with genistein concentrations as high as 13.1 µg/L [11]. In addition, fish associated with mill effluents have shown startling developmental and reproductive impairments [13-17]. Our recent research [18] investigated additional industries that could serve as sources of phytoestrogens to the environment. The waste streams from nineteen industries were sampled and analyzed for the phytoestrogens genistein, diadzein, coumestrol, formononetin, biochanin-A, and zearelenone. Eight of these industries contained phytoestrogens at what we believe to be environmentally-relevant levels (≥1000 ng/L), with the highest at about 250,000 ng/L. The influent and effluent streams of three municipal wastewater treatment plants receiving flow from some of these industries were also sampled and analyzed for the same phytoestrogens. It appeared that aerobic biological treatment, such as activated sludge, was able to remove these compounds from the liquid stream. Nevertheless, the effluent stream from one of the wastewater treatment plants had a phytoestrogen concentration above 1000 ng/L. These results indicate that phytoestrogens may be discharged from a number of industries and municipal wastewater treatment plants serving these industries. Because phytoestrogens were seen to degrade across municipal wastewater treatment plants, it is also likely that they will be degraded in surface water

as a result of biological or chemical reactions or sorption. It is currently unknown, however, how readily or quickly these reactions may occur, leaving us with a very incomplete picture of the potential environmental impact of phytoestrogens. Before we can make decisions regarding where one might want to improve treatment to remove phytoestrogen loading to surface water, we must understand more about their persistence in the environment.

3. Hypothesis

We hypothesize that photolysis and aerobic biodegradation will significantly decrease the concentrations of the phytoestrogens genistein and daidzein in surface water downstream of industrial discharges. Nevertheless, in some cases sufficient concentrations of phytoestrogens will be present to cause physiological and behavioral changes in adult and larval fathead minnows.

4. Methodology

Identify and quantify the processes that dictate the fate of genistein and daidzein in the environment

The *goal* of this objective is to quantify the processes that influence persistence so that they can be modeled; the modeled results will be verified with stream data and included in the fish exposure studies to predict the impacts of these compounds.

Aerobic Biodegradation: Biodegradation experiments will be conducted using river water samples collected from locations downstream of two soy-processing facilities in Minnesota (in Mankato and Brewster). Reactors will be constructed from serum bottles (160 mL) containing 100 mL of water and 60 mL of headspace so that sufficient oxygen is available for the duration of the experiment. Genistein and daidzein (singularly and together) will be added at a range of initial concentrations (likely ~100 ng/L to 100,000 ng/L), which is similar to those levels found exiting industries and wastewater treatment plants. For each compound at each concentration, a series of replicate bottles will be prepared. Replicate bottles will be sacrificed for liquid chromatography/mass spectrometry (LC/MS) or high performance liquid chromatography (HPLC) analysis (depending on the concentration) over time. Prior to sacrifice, the dissolved oxygen and pH of the reactor will be measured and 1 mL of solution will be removed for total organic carbon (TOC) and protein analysis. Protein will be used to quantify biomass levels and will be used to normalize the calculated degradation rate constants. Experiments will be performed over a range of dissolved oxygen and biomass concentrations as well. Expected products (biochanin A and formononetin) will also be monitored. If unknown compounds are observed to form, we will also attempt their identification. The estrogenicity of any by-products produced will be determined via the yeast estrogen screen (YES) assay, a method that uses a recombinant yeast strain to determine binding to the human estrogen receptor. We have a great deal of experience using the YES assay in our lab. All data fitting and statistical analyses will be performed using commercially available software packages (e.g., *Scientist* for Windows, Microsoft

Excel). Controls will consist of river water samples not spiked with the target compounds (for TOC and protein) or ultrapure water spiked with the target compounds (abiotic control).

Photolysis: The UV-visible absorbance spectra of genistein and daidzein will be measured in water to determine if the species are subject to direct photolysis (i.e., light of $\lambda > 290$ nm is absorbed). Each target has acid/base chemistry, so the pK_a values will be determined spectrophotometrically and absorbance spectra of the acidic and basic forms will be recorded. For example, genistein has three pK_a values, and thus pH will have an important role in light absorption rate. Experiments will be conducted using both artificial and natural light at various pH values. Initial concentrations will be low enough so that solutions can be considered optically dilute. Natural light experiments will be conducted outside on clear days, with date, time, and temperature recorded so that the solar spectrum can be accurately estimated. Artificial light will be provided by a solar simulator. Light intensity will be quantified using chemical actinometry (*p*-nitroanisole or *p*-nitroacetophenone with pyridine). By comparing the loss rates of the target compound and the actinometer, the quantum yield for parent compound loss can be determined [19]. Some pharmaceuticals are also known to bind metal ions, such as the calcium and magnesium in natural waters. Thus, experiments will also be conducted to determine whether the presence of these species influences the photolysis rates of genistein and daidzein. If this is found, metal-ligand titrations will be performed to determine appropriate binding constants. UV/Vis spectra of the complexes will be determined, and photolysis under a range of pH/metal concentrations will be performed. The estrogenicity of products will be determined using the YES assay.

The rates of indirect photolysis processes (reactions with singlet oxygen, hydroxyl radical, or excited organic matter) will be also be determined. The reaction rate constant between singlet oxygen and genistein or daidzein will be measured using a solar simulator or natural sunlight in the presence of a singlet oxygen sensitizer. The rate of target compound disappearance will be compared to a standard, furfuryl alcohol. Hydroxyl radical rate constants will be determined using Fenton's reagent. Reactors will contain a solution of target compound (~100 μM), acetophenone (~100 μM), Fe^{2+} (0.2 mM), and hydrogen peroxide (5 mM) adjusted to pH 3 with sulfuric acid. Samples (0.5 mL) will be withdrawn at predetermined intervals, quenched, and quantified. Lastly, the importance of triplet (excited) organic matter as an oxidant will be tested by adding a triplet quencher (isoprene) and sparging oxygen from reactors. In reactors containing isoprene the observed loss rate is suppressed if triplet organic matter reacts with the target compound. Rates are accelerated in the absence of oxygen, which is itself a triplet quencher. The indirect mechanisms will be tested in natural water samples, by performing experiments containing a singlet oxygen quencher (i.e. azide), or a radical quencher (tert-butanol). In the natural water experiments, we will also measure nitrate concentrations to allow estimation of the importance of nitrate in the generation of hydroxyl radicals. Using a probe molecule (p-chlorobenzoic acid) we will be able to estimate the steady-state hydroxyl radical concentration in the irradiated water samples. In all experiments, controls will consist of reactors prepared and treated identically, but wrapped in aluminum foil to prevent exposure to light. Quantification of genistein and daidzein will be performed using HPLC. Again, the estrogenicity of the products will be determined using the YES assay.

Sorption: Water-solid partitioning coefficients will be determined for each compound in batch equilibrium experiments. A known mass of either genistein or daidzein will be added to a serum bottle with known volumes of sterile water and sterile solid material [20]. The solids will be sterilized by gamma-irradiating them. Vials will be wrapped in aluminum foil to avoid photolysis. Once equilibrium is reached, the concentration in the aqueous phase will be determined via HPLC, the concentration in the solid determined by difference, and the partition coefficients will be calculated. Because the compounds have acid-base chemistry, this will be performed at five different pH values. Three solids will be tested 1) kaolinite clay, 2) sand, and 3) river sediments collected downgradient of the wastewater discharges. Sediment samples will be submitted to the University of Minnesota Soil Testing Laboratory for determination of organic carbon content and other characterization.

Field measurements: Triplicate samples (5 L each) will be taken downstream of the two soy-processing facilities mentioned above (5 sampling locations) under different seasonal conditions (spring, summer, fall, and winter, see timetable below). Samples will be processed and phytoestrogens, protein, total organic carbon, pH, and temperature will be measured. Temperature and pH will be measured in the field with a thermometer and a portable pH probe. Protein and total organic carbon will be measured after returning to the lab using the Lowry method and an Organic Carbon Analyzer (Tekmar-Dohrmann). Genistein and daidzein will be measured by first filtering the samples with a glass fiber filter, then extracting the phytoestrogens into methanol using solid phase extraction (C_{18}, 60 mL ChromTech Resprep). The eluted methanol will be volume-reduced and analyzed by LC/MS in selected ion mode.

Field modeling: After quantifying the appropriate rate constants, verification of the importance of these processes in the field is required. Based on the known discharge concentrations (measured), the dilution upon discharge into the river (information obtained from the facilities, nearby US Geological Survey river/stream gauge measurements, or in-stream velocity and depth measurements taken at the time of sample collection), the measured rate constants for biodegradation, photolysis, and sorption to/settling of particles, and required environmental parameters (i.e. flow rate, solar exposure, etc.), a model for the concentration of the appropriate targets as a function of distance will be built based on those described in Schwarzenbach et al. [20]. Sensitivity analysis of the results will be performed by varying the rate constants to see which have the largest effect on the model output. The model output will then be compared to measurements of genistein, daidzein, and any degradation products detected in samples taken at the point of discharge and in the rivers/streams downgradient of the two soy-processing facilities.

Hypothesized outcomes: It is expected that genistein and daidzein will be subject to biodegradation and one or more photolysis processes and that these processes will dominate their fate in the environment. It is also expected that these processes will be rapid enough to appreciably degrade the compounds downstream of discharge points. Sorption/settling may gain importance depending on the suspended sediment concentrations and charge state of the particles and chemicals. If it is found that photolysis and aerobic biodegradation cannot explain the observed losses, additional experiments to identify other possibilities (i.e. anaerobic biodegradation in the sediment,

hydrolysis, abiotic reactions mediated by suspended mineral particles [21]) will be performed.

Quantification of the impact of genistein and daidzein on fathead minnows

The *goal* of this objective is to study the effect of genistein and daidzein exposure on the survival and reproduction of fathead minnows. We chose the fathead minnow for this study as it is a tier one screening organism [22], and is a well-established laboratory model for endocrine disruption studies [23, 24, 25]. Fathead minnows will reproduce year-round in the laboratory with paternal fish engaging in nest care, which is central to reproductive fitness in this species [26, 27]. Finally, fathead minnows are ubiquitous in North American waters, providing environmental relevance.

Exposures: Initial exposure studies will be performed with genistein and daidzein singularly and in combination at concentrations consistent with those observed previously [18]. Experiments will also be performed with compounds at concentrations one order of magnitude above these levels to insure broad applicability of results. If the degradation products of daidzein and genistein can be identified and they are observed to persist for environmentally-relevant periods of time (days), exposure studies will also be performed with these compounds if they are available for purchase (formononetin, for example). Laboratory results will be field validated using *in situ* experiments with fathead minnows caged upstream and downstream of the two soy-processing facilities.

Flow-through laboratory exposure experiments will use the well-established and validated infrastructure available at the Aquatic Toxicology Laboratory at St. Cloud State University [*e.g.,* 28, 29]. Briefly, well water will be heated to 21°C and directed into an aerated head tank. Water will then be mixed with a specific amount of chemical and distributed into multiple aquaria housing the exposure organisms. Continuous monitoring of water quality, continuous monitoring of environmental conditions (temperature, photoperiod), daily monitoring of water chemistry (DO, ammonia, pH, hardness, etc.), and weekly monitoring of exposure chemical concentrations (via LC/MS or HPLC) in all treatments will assure the reliability of the experiments. Duplicate experiments will be conducted in which single-sex groups of male or female fish (n=20/treatment and sex) are exposed to three concentrations each of genistein and daidzein and mixtures of the two compounds at the same three concentrations. An ethanol carrier control experiment will also be performed. Following the 21-day exposure period half of the fish in each treatment (n=10) will be sacrificed and assessed for a suite of biological endpoints commonly associated with estrogenic exposure (see below). The remaining males and females from each treatment will be combined into single spawning pairs (n=10 per treatment) and their reproductive output (egg production, fertilized eggs, hatching success, 30 day larval survival) will be monitored. In addition, two behavioral assays will be conducted to determine whether exposed male fish are less likely to acquire and defend a nest site, which is crucial to their reproductive ability in the wild. The biological endpoints of interest are survival and reproductive ability. Survival to a reproductive age is largely the result of being able to move out of harm's way, or locomotion. Reproductive ability hinges on three factors, 1) the ability of the male to mate, 2) the female's fecundity, and 3) the survival of offspring

to reproductive age. A mobile laboratory also is available so that testing can be performed at remote locations (such as wastewater treatment plants).

In situ field exposures of mature male and female fathead minnows will be used to validate findings from the laboratory experiments. Briefly, single-sex groups of male and female fathead minnows will be caged upstream and downstream in the receiving streams of the two soy-processing facilities. At each upstream/downstream site four cages, each containing 10 males or 10 females (n=20 per site and sex, similar to laboratory exposures), will be anchored at the stream bottom in an area of low current with ample access to bottom substrate. In previous studies, we have had excellent survival (>80%) for fish caged under these conditions for 21 days (similar to laboratory exposures). Fish will be provided with slow-dissolving food pellets to substitute natural feed. After 21 days fish will be retrieved and processed in the laboratory in a similar manner to that described for laboratory exposure experiments. Briefly, half the fish (n=10 per sex and site) will be sacrificed and analyzed for proximate physiological and morphological endpoints. The remaining 10 males and females from each treatment will be placed in spawning pairs for the 10-day reproductive assay. Fecundity, fertility and larval survival to 30 days post-hatch will be recorded.

Proximate physiological and morphological endpoints: Following toxicological convention for estrogenic exposure studies, we will assess a suite of physiological and morphological endpoints in exposed male and female fathead minnows to assess the presence of a biological response in the exposed organisms. Sacrificed fish will be weighed, their total and standard length will be determined, and organs will be excised and weighed. These measurements will allow the calculation of a body condition index (weight [g]/standard length [cm^3]), an indicator of the metabolic health of the exposed fish. Furthermore, the relative weight of reproductive organs (testis or ovary) and of the liver will be calculated ((organ weight/body weight)*100). These two indices are commonly used in aquatic toxicology and provide a rough estimate for the organ-level effects of exposure. Blood will be drawn from the caudal vasculature of each fish to quantify the egg-yolk precursor protein vitellogenin, commonly considered an indicator of acute exposure to estrogenic compounds when found in male fish.

Locomotion: Offspring are under a constant threat of predation. Most larval fish respond to perceived threats through two linked actions: a quick response and a high velocity escape motion. After exposure to estrogenic compounds, juvenile fathead minnows will be subjected to a standardized perceived threat (a pressure wave caused by a rubber mallet). Their reaction will be recorded with a high-speed digital camera and the time to the initial reaction and the velocity of the evasive maneuver will be calculated. Minnows will be processed for neurochemical endpoints to assess physiological alterations that may under-lie behavioral changes [30]. Control experiments will also be performed with unexposed juvenile minnows.

Male reproductive ability: The nest holding behavior of male fathead minnows is thought to be under the control of androgen, concentrations of which are likely to decrease in the presence of estrogenic compounds [31]. Thus, male reproductive inadequacy is not only selected against in direct competition with other males, but also by the prolonged need to defend a nest site. We have determined through DNA analysis

that only the male holding the nest site produces offspring [32]. Male fathead minnows will be exposed to genistein and daidzein for 21 days and then paired with a female fathead minnow in a small tank containing only one nest site for the ensuing 10 days. During this time, a plastic model of a male fathead minnow will be introduced into the aquarium on three occasions to elicit a defense response by the nest-holding male fathead minnow in the aquarium. The nest-defense behavior is under androgenic control and may be diminished in response to the estrogenic exposure of the male prior to the aggression assay. Time to attack (usually <30 sec in unexposed males) and number of attacks against the perceived intruder (plastic male minnow) in the ensuing 60 sec (often >10) will be recorded.

Female fecundity: Female fathead minnows are not involved in reproductive activities beyond laying eggs. This will allow us to adapt experimental designs developed and validated by the US EPA [23, 24]. Briefly, we will expose mature female fathead minnows for 21 days to genistein and daidzein before placing them into an aquarium with a male fish and a nest site. Egg production will be monitored daily across multiple ovulation cycles to obtain information on how many eggs are produced over time and the time between egg-laying events. In addition, fertilization, hatching, and 10-day survival rates for F1 generation offspring will be recorded.

Offspring survival: Offspring from the two experiments described above will be maintained until 30 days post-hatch (a time point at which sexual differentiation has occurred). Once mature, the male reproductive ability and female fecundity of the offspring will be assessed. This will allow us to determine reproductive success across two generations.

Hypothesized outcomes: It is expected that phytoestrogen exposure at environmentally-relevant concentrations will reduce reproductive fitness in exposed fathead minnows through several avenues. First, phytoestrogen-exposed male fathead minnows will exhibit reduced reproductive behaviors and nest defense, resulting in lower hatching success per nest site. Second, phytoestrogen-exposed female fathead minnows will become arrhythmic in their ovulation cycle (usually every 4-5 days) and therefore exhibit decreased fecundity. Third, phytoestrogen-exposed juvenile fathead minnows will exhibit higher predatory mortality due to their diminished capacity to detect and evade predators using their innate escape behaviors. Results from these exposure studies can be integrated in existing population dynamics models [33] to determine the population level, and ultimately trophic effect of phytoestrogen exposure.

Quality assurance and quality control

Standard quality assurance/quality control (QA/QC) protocols will be used throughout all experimental portions of the project, including surrogate and internal standard comparison, calibration curves containing a minimum of seven points, and routine analysis of blanks (ultrapure laboratory water) and spiked samples. Precision and accuracy will be evaluated via replicate experiments and analyses. Additionally, errors (95% confidence intervals) associated with instrument calibration and detection limits will be quantified. All measured concentrations will be reported with appropriate propagated errors. Fish exposure and analysis protocols follow well established

guidelines [25]. Briefly, exposures are conducted in triplicate with high sample sizes (>20 animals/treatment). Tissue collections follow established USGS BEST practices [34]. Sample evaluation is conducted by trained scientists using a blind analysis scheme with evaluation replication to assure consistency. Fish data exceeding 5% variability are re-evaluated

5. Results and Deliverables

As mentioned above, the expected outcomes of this project are two-fold. First, we expect to gain an understanding of how genistein and daidzein degrade in the environment and at what rate this occurs. This will be based on laboratory experiments, modeling, and field measurements. The deliverable for this outcome will be the rate constants associated with sorption, biodegradation and photolysis and a model that predicts the loss rate of the phytoestrogens as a function of distance from the discharge point. Second, we will determine how an ecologically important fish species, the fathead minnow, is affected by a range of phytoestrogen concentrations. We expect that in some cases discharges of phytoestrogens will be high enough that, even with degradation occurring in the environment, the reproductive fitness of minnows will be reduced, causing potential population-level (and therefore food-chain) impacts. In other instances, however, the degradation of these compounds in the natural environment will be sufficient to limit their impact on minnows. The deliverable for this task is an evaluation as to whether phytoestrogens discharged in wastewaters pose a risk to Minnesota fish. We expect to be able to roughly predict when phytoestrogen discharge may be a problem and offer recommendations regarding where additional wastewater treatment may be needed.

6. Timetable

This project is a three-year project, beginning in July, 2010. 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**
Field samples taken	**X**		**X**			**X**		**X**				
Biodegradation experiments		**X**	**X**	**X**	**X**	**X**	**X**	**X**				
Direct Photolysis experiments	**X**	**X**	**X**									
Indirect Photolysis experiments	**X**	**X**	**X**	**X**	**X**							
Sorption experiments						**X**	**X**	**X**				
Direct Photolysis experiments			**X**	**X**	**X**							
Indirect Photolysis experiments					**X**	**X**	**X**	**X**				
Sorption experiments		**X**	**X**	**X**								

Model creation								X	X	X	X	
Model verification with field samples (taken at a variety of times/temperatures/sunlight intensities, see above)										X	X	
Embryonic and larval fathead minnow experiments				X	X	X	X					
Mature fathead minnow reproduction experiments				X	X	X	X	X				
Caged minnow studies								X	X			
Physiological analysis of exposed mature fathead minnows						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 budget is as outlined on the previously submitted proposal (see Attachment A). A budget justification is provided below.

Personnel

Over the course of the 3-year project support for two graduate students and three of the PIs is budgeted. One of the PIs (Heiko Schoenfuss) is employed by St. Cloud State University and his salary will be covered under a subcontract (see below). The PI (Novak) and Co-PI Arnold will each receive 2 weeks of salary a year for the first 2 years and then 3 weeks for the last year. Schoenfuss will receive 2 weeks of salary for years 1-2, but no salary in year 3 as a result of teaching obligations in that year. Fringe benefits for the PIs at UMN are set at 32.3% by the University of Minnesota. Schoenfuss' fringe benefit rate is 27%. The PIs will be responsible for project oversight, guidance of the graduate students, 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, plus 2 summer, period. Fringe benefits for graduate students include tuition, health insurance (estimated at 17.56% of salary), and summer FICA (7.44% of salary). We anticipate that these two graduate students will continue to work on the project for all 3 years, and that this additional effort will be supplemented by teaching assistantships obtained through the University of Minnesota. One undergraduate research assistant ($4,000/year for 3 years) will be employed to perform the fish studies. This employee will reside at St. Cloud State University and will be part of the subcontract (see below)

Materials and Supplies
Funds ($74,539) 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, quartz test-tubes and bottles, analytical consumables, analytical fees, solvents, reagents, fish, gloves, digital data storage media, and laboratory notebooks. A portion of this (see below, $35,502) will be part of the subcontract to St. Cloud State University.

Travel
Travel funds are included for the project for travel to obtain samples and monitor in-stream experiments. A portion of these funds (see below, $1,125) will be part of the subcontract to St. Cloud State University.

Subcontract
St. Cloud State will receive funds to support Dr. Schoenfuss' time and effort ($9,373), an undergraduate research assistant ($12,000 over 3 years), materials and supplies ($35,502 over 3 years), and travel funds ($1,125 over 3 years). These funds are all justified above. This work will be completed at St. Cloud State University with the equipment there.

Total amount proposed
The total proposed project amount is $340,000. No indirect costs for the University of Minnesota are included in the budget.

8. Credentials

Paige J. Novak

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.

Selected Publications
Henjum, M. B., Hozalski, R. M., Wennen, C. R., Arnold, W. A., Novak, P. J. 2009. Correlations between In Situ Sensor Measurements and Trace Organic Pollutants in Urban Streams. *Journal of Environmental Monitoring*, in press.

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

Nelson, D. K., Novak, P. J. 2009. Enhanced Dissolution of Trichloroethene: Effect of Carbohydrate Addition and Fermentation Processes. *Journal of Environmental Engineering*, 135:861-868.

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.

Yan, T., LaPara, T. M., *Novak, P. J. 2006. The Reductive Dechlorination of 2,3,4,5-Tetrachlorobiphenyl in Three Different Sediment Cultures: Evidence for the Involvement of Phylogenetically Similar *Dehalococcoides*-like Bacterial Populations. *FEMS Microbiology Ecology*, 55(2):248-261.

William Arnold

B.S., Chemical Engineering, 1994, Massachusetts Institute of Technology, Cambridge, MA.
M.S., Chemical Engineering, 1995, Yale University, New Haven, CT.
Ph.D., Environmental Engineering, 1999, The Johns Hopkins University, Baltimore, MD.

Research

Major research efforts focus on the fate of organic chemicals in natural and engineered aquatic systems. Specific research areas include studying the kinetics, pathways and mechanisms of anthropogenic chemical reactions that occur at surfaces or via photochemical processes; evaluating mass transfer effects on reaction rates; developing new remediation/containment techniques; and using computational chemistry techniques to predict and/or explain experimental observations. Current projects are investigating pharmaceutical fate in aquatic systems.

Selected Publications

Buth, J.M., McNeill, K., Arnold, W.A. 2009. Aquatic photochemistry of chlorinated triclosan derivatives: potential source of polychlorodibenzo-p-dioxins. *Environmental Toxicology & Chemistry,* accepted pending minor revisions.

Arnold, W.A.**,** Bolotin, J., von Gunten, U., Hofstetter, T.B. 2008. Evaluation of functional groups responsible for chloroform formation during water chlorination using compound specific isotope analysis, *Environmental Science and Technology,* 42(21), pp. 7778–7785.

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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 Publications
Hyndman, K.M., Biales, A., Bartell, S.E., Schoenfuss, H.L. Accepted. Assessing the effects of exposure timing on biomarker expression using 17ß-estradiol. *Aquatic Toxicology*.

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.

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9. Dissemination and Use

The target audience for results from this research will be professionals in the area of wastewater treatment, watershed management, and industry. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the MDA 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. Results will be used to target when, where, and how to best to treat industrial wastewater streams that contain high concentrations of phytoestrogens so that aquatic organisms are adequately protected.

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