2010 Project Abstract
For the Period Ending June 30, 2013

PROJECT TITLE:	Ecological Impacts of Effluent in Surface Waters and Fish
PROJECT MANAGER:	Paige J. Novak, Ph.D., P.E.
AFFILIATION:	University of Minnesota, Department of Civil Engineering
MAILING ADDRESS:	122 Civil Engineering, 500 Pillsbury Dr. SE
CITY/STATE/ZIP:	Minneapolis, Minnesota 55455
PHONE:	(612) 626-9846
FAX:	(612) 626-7750
E-MAIL:	novak010@umn.edu
WEBSITE:	N/A
FUNDING SOURCE:	Minnesota Environment and Natural Resources Trust Fund
LEGAL CITATION:	M.L. 2010, Chp. 362, Sec. 2, Subd. 5c

APPROPRIATION AMOUNT: $340,000

Overall Project Outcome and Results

Phytoestrogens are plant-based compounds that mimic estrogen and can interfere with normal biological development. Research shows that phytoestrogens are discharged into surface water from wastewater treatment plants and certain industries. The biological effects of these compounds have not been well studied, although it is known that they can feminize male fish. Almost nothing is known about their environmental fate. When these compounds enter rivers and streams, it is likely that they will be degraded and therefore may have a lessened impact on biota, but this needs to be confirmed

In this project, the persistence of two common phytoestrogens (genistein and daidzein) was studied. Fathead minnow exposure experiments at realistic environmental concentrations were also performed. Experiments demonstrated that genistein and daidzein reacted with sunlight. These two compounds also biodegraded rapidly in natural water samples; the rate of degradation depended on phytoestrogen concentration, water/incubation temperature, and the source of the water. Sorption experiments showed that phytoestrogens sorb to sediment, but this is not likely to be an important loss mechanism. Adult fathead minnow exposure experiments showed that only subtle effects on anatomy, physiology and behavior of fathead minnows occurred as a result of exposure to phytoestrogens singly or in mixtures. The one exception to this was the fact that adult fathead minnows produced significantly more eggs when exposed to daidzein. Larval minnow exposures showed that exposure to genistein, formononetin (another common phytoestrogen), and a mixture of phytoestrogens had a negative impact on larval survival. Adult and larval exposures to microbiologically degraded phytoestrogens showed negative impacts on adult egg production. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates.

Project Results Use and Dissemination

Results have been disseminated at several conferences. In addition, one manuscript has been published, two additional manuscripts have been submitted, and a fourth is being revised and will be submitted for publication in August or September, 2013. This project also resulted in the generation of two Master's theses and one Ph.D. thesis.

2010 Environment and Natural Resources Trust Fund (ENRTF) Work Program Final Report

Date of Report: August 22, 2013
Final Report
Date of Work Program Approval: June 9, 2010
Project Completion Date: June 30, 2013

I. PROJECT TITLE: Ecological Impacts of Effluent in Surface Waters and Fish

Project Manager: Paige J. Novak, Ph.D., P.E.
Affiliation: University of Minnesota, Department of Civil Engineering
Mailing Address: 122 Civil Engineering, 500 Pillsbury Dr. SE
City / State / Zip: Minneapolis, Minnesota 55455
Telephone Number: (612) 626-9846
E-mail Address: novak010@umn.edu
FAX Number: (612) 626-7750
Web Page address: N/A

Location: Minneapolis, Minnesota 55455 or St. Cloud, Minnesota 56301; Additional work (sampling) will take place in Mankato, Minnesota and Brewster, Minnesota. See attached map.

Total ENRTF Project Budget:	**ENRTF Appropriation $**	**340,000**
	Minus Amount Spent: $	**340,000**
	Equal Balance: $	**0**

Legal Citation: M.L. 2010, Chp. 362, Sec. 2, Subd. 5c

Appropriation Language:
$340,000 is from the trust fund to the Board of Regents of the University of Minnesota in cooperation with St. Cloud State University to determine the chemical and biological fate of phytoestrogens in surface waters and the impacts on fish. This appropriation is available until June 30, 2013, by which time the project must be completed and final products delivered.

II. FINAL PROJECT SUMMARY AND RESULTS:

Phytoestrogens are plant-based compounds that mimic estrogen and can interfere with normal biological development. Research shows that phytoestrogens are discharged into surface water from wastewater treatment plants and certain industries. The biological effects of these compounds have not been well studied, although it is known that they can feminize male fish. Almost nothing is known about their environmental fate. When these compounds enter rivers and streams, it is likely that they will be

degraded and therefore may have a lessened impact on biota, but this needs to be confirmed

In this project, the persistence of two common phytoestrogens (genistein and daidzein) was studied. Fathead minnow exposure experiments at realistic environmental concentrations were also performed. Experiments demonstrated that genistein and daidzein reacted with sunlight. These two compounds also biodegraded rapidly in natural water samples; the rate of degradation depended on phytoestrogen concentration, water/incubation temperature, and the source of the water. Sorption experiments showed that phytoestrogens sorb to sediment, but this is not likely to be an important loss mechanism. Adult fathead minnow exposure experiments showed that only subtle effects on anatomy, physiology and behavior of fathead minnows occurred as a result of exposure to phytoestrogens singly or in mixtures. The one exception to this was the fact that adult fathead minnows produced significantly more eggs when exposed to daidzein. Larval minnow exposures showed that exposure to genistein, formononetin (another common phytoestrogen), and a mixture of phytoestrogens had a negative impact on larval survival. Adult and larval exposures to microbiologically degraded phytoestrogens showed negative impacts on adult egg production. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates.

III. PROGRESS SUMMARY AS OF JANUARY, 2013:

Photolysis experiments with genistein and daidzein are complete and this work has been published. Biodegradation experiments are also complete and showed that genistein degradation was rapid, variable (based on initial concentration, temperature, and source water), and followed zero-order kinetics at high initial concentrations (100 µg/L) and first-order kinetics at lower initial concentrations (500 ng/L). Half-lives varied from about 18.5 to 53 hours. Although degradation was relatively rapid, phytoestrogens are likely to be more persistent in cold weather months from discharges to lower-flow streams, such as Okabena Creek. Laboratory-scale reactor experiments showed that although genistein degradation occurred under nitrifying conditions (low carbon, long residence time, high ammonia concentration), nitrifiers were not responsible for the transformation. This points to genistein degraders as multiple substrate utilizers that may be slow growers. Grab samples collected in the Minnesota River up- and downstream of the Mankato WWTP November 2011, May 2012, and June 2012 for showed that any phytoestrogens present in the effluent were fully diluted by the river upon discharge. Like the Minnesota River samples, samples collected up- and downstream of the Brewster WWTP in June of 2012 showed that the effluent did not impact Okabena Creek water quality with respect to phytoestrogens; no phytoestrogen signature from the Brewster WWTP discharge was discernable. Sorption isotherms for genistein, daidzein, and formononetin to sediment collected from the Mississippi River were determined, as were isotherms for the sorption of genistein and biochanin A to

kaolinite and Na- and Ca-montmorillonite at a range of pH values. Preliminary modeling to understand some of the mechanisms of sorption was also performed. Adult fathead minnow exposure experiments suggested only subtle effects (on anatomy, physiology and behavior of mature fathead minnows) as a result of exposure to phytoestrogens singly or in mixtures. Larval exposures, however, showed that genistein, daidzein, a mixture of phytoestrogens, sediment-sorbed genistein, and sediment-sorbed formononetin all had negative impacts on larval survival, although no impacts on larval predator avoidance were observed.

Amendment Request (01/20/2012):
The addendum is to formally request a re-budgeting of funds for this project.

As part of the project, an accelerated solvent extraction system is used to extract phytoestrogens from sediment for modeling and sorption studies. A valve in the system has broken and needs to be replaced. While the part is not under warranty, we have negotiated a reduced cost for the repair, because this is something that should not have failed so soon. The total cost of the repair has been quoted at $2,300. Based on usage to date, 70% of the repair ($1610) will be charged to this project (the remaining funds for the repair will be obtained from a requested re-budget of Prof. William Arnold's LCCMR project, M.L. 2010 5f - Evaluation of Dioxins in Minnesota Lakes, which co-purchased and is co-using the instrument). We would like to rebudget $2,000 from the "**Personnel**" category to the "**Equipment/Tools/Supplies**" category where "and instrument maintenance and repair costs" has been added. The additional funds being re-budgeted are insurance against any future repairs.

In addition, personnel requirements for Result 2 (Determine the impact of the phytoestrogens on fathead minnows) are less than originally anticipated because undergraduate students have been able to complete more of the work that was proposed. The supply costs for determining phytoestrogen concentrations in rivers downstream of the wastewater treatment plants are higher than originally anticipated because of the low concentrations of these compounds as a result of dilution from the high water levels this past spring and summer. Because of this a rebudget of $15,000 is requested from the "**Personnel**" category to the "**Equipment/Tools/Supplies**" category.

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 01/24/2012

Amendment Request (07/31/2012):
The addendum is to formally request a re-budgeting of funds for this project.

Result 2 is primarily being conducted at St. Cloud State University under the direction of Heiko Schoenfuss (co-investigator). A graduate student hired through the University of Minnesota has been working on Result 2, but an additional student with specific training

in histological methods was required to complete this research. Therefore, we would like to rebudget $24,000 from the "**Personnel**" category (student support for Result 2) to the "**Subcontract**" category, where it will be used for the exact same purpose (student support for Result 2) with the only difference being that the student will attend St. Cloud State University rather than the University of Minnesota.

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 08/2/2012

Amendment Request (04/3/2013):
The addendum is to formally request a re-budgeting of funds for this project.

The supply costs for determining phytoestrogen concentrations in rivers and performing experiments on the biological and chemical fate of phytoestrogens have been higher than originally anticipated because of the low concentrations of these compounds and the large number of experiments performed. This has resulted in additional analytical and material costs. Because of this a rebudget of $14,000 is requested from the "**Personnel**" category (Result 2) to the "**Equipment/Tools/Supplies**" category (Result 1).

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 04/5/2013

IV. OUTLINE OF PROJECT RESULTS:

RESULT 1: *Determine the chemical and biological fate of phytoestrogens in surface waters*

Description:
We know that phytoestrogens are discharged to surface water from municipal wastewater treatment plants and from industrial facilities (including some dairies, meat processors, peanut processors, and soy processors). Nevertheless, no research has been conducted on their biological or chemical fate in the environment. It is likely that these compounds will adhere to particles in the receiving water and will undergo chemical and biological reactions. These processes will control the concentration of the phytoestrogens, and therefore, their ecological effect (see Result 2, below). Laboratory experiments will be performed with the two most-commonly observed phytoestrogens: genistein and daidzein. Single compounds and mixtures will be added to river water samples collected downstream of two soy-processing facilities in Minnesota (in Mankato and Brewster). The biological transformation of the phytoestrogens will be measured with time using liquid chromatography/mass spectrometry under different conditions

(phytoestrogen concentration, biomass levels, oxygen levels). The estrogenicity of any byproducts formed will be determined as well, using a yeast estrogen screen assay. Experiments to determine photolysis (sunlight-driven reactions) rates of genistein and daidzein will also be conducted in river and pure water in both artificial and natural light. The effect of naturally-occurring ions and organic matter on photolysis rates will be investigated. Again, the estrogenicity of the byproducts will be determined. Finally, the water-solid partitioning coefficients will be determined for both compounds.
After quantifying the appropriate rate constants, verification of the importance of these processes in the field is required. We will determine the concentration of genistein and daidzein at the point of discharge and in the rivers/streams downgradient of the two soy-processing facilities. A model for the concentration of phytoestrogens as a function of distance will be built, based on our experimental results, and compared to the concentrations measured in the field.

Summary Budget Information for Result 1:	**ENRTF Budget:**	**$**	**220,000**
	Amount Spent:	**$**	**220,000**
	Balance:	**$**	**0**

Deliverable/Outcome	**Completion Date**	**Budget**
1. Determine the biological transformation kinetics for genistein and daidzein	6/30/12	62,000
2. Determine kinetics of photolysis for genistein and daidzein	6/30/12	56,000
3. Determine estrogenicity of transformation products of genistein and daidzein	6/30/12	21,000
4. Measure the effluent concentrations and downgradient concentrations of genistein and daidzein in the field	9/30/12	40,000
5. Build and verify a model to determine the importance of various natural processes on phytoestrogen fate in the environment	4/30/13	41,000

Result Completion Date: April 30, 2013

Result Status as of January 2011:
The UV-visible absorbance spectra of genistein and daidzein have been measured and pK_a values have been determined. Genistein and daidzein have been found to undergo direct photolysis, and the quantum yields of direct photolysis for genistein and daidzein have been determined using natural sunlight and chemical actinometry (see Table 1 below). The direct photolysis of daidzein is unaffected by calcium and magnesium; the effect of these cations on genistein photolysis is currently being investigated.

Table 1. Quantum Yields of Direct Photolysis of Genistein and Daidzein

Genistein	Φ	Daidzein	Φ
pH 5	0.000003	pH 5	0.00043

pH 8.5	0.000038	pH 8.7	0.001
pH 11	0.000044	pH 12	0.00019
pH 12	0.00114		

Indirect photolysis for both compounds is currently being investigated as well. The rate constant for the reaction between daidzein and hydroxyl radical has been determined to be $1.01*10^{10}$; genistein has not yet been investigated.

Several biodegradation experiments have been performed with surface water that is unimpacted by soy-processing facilities. The biodegradation rates of genistein and daidzein are extremely variable in surface water, with 90% of the parent compound degrading in approximately 2-7 days. The rate of degradation appears to depend on several factors including water temperature, biomass concentration, water turbidity, and phytoestrogen concentration. Additional experiments are currently being performed to better-understand the influence of various environmental parameters. Experiments using surface water from soy processing-impacted areas at Brewster and Mankato, MN are planned for this summer.

Result Status as of July 2011:

The reaction rates between genistein and daidzein and reactive oxygen species (ROS) have been determined (see below).

Rate constants for reactions between genistein and daidzein and ROS

	k_{OH} (M^{-1} s^{-1})	K_{1O2} (M^{-1} s^{-1})
Genistein	8.73×10^{9}	3.57×10^{7}
Daidzein	~~1.01×10^{10}~~ 6.9×10^{9}	1.84×10^{7}

Sampling downgradient of the Mankato wastewater treatment plant (WWTP) was postponed until late June because of high water. On June 20th the sampling trip was made and samples were taken to perform biodegradation and sediment sorption experiments in the laboratory at the University of Minnesota. Samples were also taken to monitor the attenuation of phytoestrogens downstream from their input at the WWTP effluent discharge.

Experiments designed to understand the sorption of genistein and daidzein to solid matrices have also begun using both natural sediments (collected from the Minnesota River upstream of the Mankato WWTP) and model solids (*e.g.,* kaolinite). Preliminary data suggests that genistein and daidzein do not sorb appreciably to kaolinite. Additional experiments will explore the effect of kaolinite concentration on sorption. Preliminary experiments performed with the river sediment show that sorption increases as pH decreases.

Biodegradation experiments were performed with the Mankato surface water. In order to begin to study those environmental parameters that impact degradation, experiments were performed with varying initial genistein concentrations (150 ng/L, 1 μg/L and 100

μg/L). Dissolved oxygen and pH were also monitored. Degradation of genistein was rapid and zero order over this range of concentrations, with genistein dropping below the detection limit (1.5 ng/L) within 70 hours.

Twenty-five grab samples were collected from the Mankato WWTP effluent and at select locations downstream of the wastewater outfall. These samples were collected to determine the effects of dilution/degradation on the concentration of various phytoestrogen species. A method is currently being refined on the LC-MS/MS to analyze for genistein, diadzein, zearalenone, formenonetin, coumestrol, and biochanin A at the Masonic Cancer Center.

Result Status as of January 2012:

Grab samples collected during the June 20th, 2011 sampling trip were analyzed for six phytoestrogens: zearlenone, daidzein, coumestrol, formenonetin, genistein and biochanin A. Samples collected upstream of the Mankato WWTP yielded the following phytoestrogen concentrations: zearlenone (1.1 ng/L), daidzein (1.8-1.5 ng/L), formenonetin (2.4-1.9 ng/L), genistein (1.8-1.6 ng/L) and biochanin A (1.9-1.6 ng/L). Analysis of the downgradient samples showed no impact from the Mankato WWTP on phytoestrogen concentrations in the Minnesota River (essentially the same concentrations of phytoestrogens were detected upstream and downstream of the wastewater treatment plant). It is likely that this is a result of the Minnesota River being near flood stage (22 ft) during sampling and the subsequent dilution of the wastewater effluent that occurred. The high water level also made collecting a direct WWTP effluent sample impossible (the outlet was under the water level) and therefore the degree to which the effluent was diluted by the river could not be determined. The closest sample was collected within 10 ft of the effluent pipe and the furthest downstream sample collected was approximately 0.6mi downstream of the WWTP.

A second sampling trip was conducted in November 2011. Thirteen grab samples were collected from the Minnesota River at Mankato (1 upstream, 1 effluent, and 11 downstream samples) and 10 samples were collected up- and downstream of the Brewster WWTP. These samples have been prepared for analysis by LC-MS/MS, which will take place in March at the Masonic Cancer Center at the University of Minnesota. Water was also collected from both locations to conduct biodegradation experiments in the laboratory.

Batch genistein biodegradation experiments were conducted using water collected from the Minnesota River at Mankato (collected in November 2011). Genistein was added to the water at concentrations of 100 μg/L and 150 ng/L and degradation was monitored over time. Degradation of genistein when added at 100 μg/L was zero-order, with reaction rate coefficients of 0.0644-0.0183 μg/L-hr. Analysis of the genistein degradation at 150 ng/L study is on-going. Biodegradation experiments will be conducted at 10°C using Minnesota River water to determine the effect of temperature on biodegradation rates. Experiments will be conducted with the water collected from Brewster following an identical protocol.

The photolysis experiments have been completed and this work has been submitted to *Environmental Science & Technology* for publication. No effect of calcium or magnesium ions on genistein photolysis was seen. It was shown that reaction with triplet-state natural organic matter is the major photolytic reaction pathway for genistein and reaction via direct photolysis and with singlet oxygen are the major photolytic reaction pathways for daidzein. Additionally, the second-order rate constant for the reaction of daidzein with hydroxyl radical was determined to be 6.9×10^9 $M^{-1}s^{-1}$ (note, this value was incorrectly reported in the July 2011 report, now corrected with a mark-out). Under certain conditions where hydroxyl radical is present in high concentrations, reaction with hydroxyl radical could be important for both compounds. Subject only to direct and indirect photolysis, genistein is expected to have a half-life on the order of days to weeks, while daidzein is expected to have a half-life on the order of hours to days.

Sorption experiments are ongoing. It was shown that both compounds do sorb to kaolinite, but the amount sorbed varies with the initial concentration. It was also shown that pH affects the amount sorbed over a range of initial concentrations. Cation bridging and electrostatic interactions are expected to be important mechanisms of sorption to clays, and experiments are being conducted to assess their importance for sorption to kaolinite and montmorillonite. Sorption experiments with iron oxides are also planned.

Result Status as of July 2012

Grab samples collected upstream and downstream of the Mankato and Brewster WWTPs during the November 2011 sampling trip were analyzed. Samples collected upstream of the Mankato WWTP yielded the following phytoestrogen concentrations: zearlenone (1.1-1.2 ng/L), daidzein (1.8-1.5 ng/L), formenonetin (2.4-1.9 ng/L), genistein (2.6-1.7 ng/L) and biochanin A (2.3-1.7 ng/L). Analysis of the downgradient samples showed no impact from the Mankato WWTP on phytoestrogen concentrations in the Minnesota River (essentially the same concentrations of phytoestrogens were detected upstream and downstream of the wastewater treatment plant). Again, although not at flood stage, it is likely that the effluent is sufficiently diluted by the Minnesota River such that no impact of the wastewater effluent on the river is seen. Interestingly, these values are very similar to that seen in June 2011, suggesting that the background phytoestrogen concentrations in the Minnesota River at Mankato are fairly stable. Unfortunately, during processing, the Brewster samples were lost and could not be analyzed. Samples were also taken June 6th, 2012 (13 at Mankato and 12 at Brewster) and have been processed for analysis. These samples will be analyzed on the LC-MS/MS at the Masonic Cancer Center (University of Minnesota) in August (2012).

Experiments to determine the sorption isotherms and edges for genistein and daidzein to kaolinite, montmorillonite, and goethite, three common soil minerals, have been performed. The isotherms allowed the calculation of the distribution coefficient, K_D, for each combination, which are shown below.

K_D values (L/kg, with 95% confidence intervals) for phytoestrogens on soil minerals

	Ca-montmorillonite (pH 8.5)	Na-montmorillonite (pH 8.5)	Kaolinite (pH 8.5)	Goethite (pH 7.1)
Genistein	--	130 ± 220	64 ± 15	253 ± 60
Daidzein	40 ± 16	--	44 ± 31	37 ± 8

The edge experiments, in which the concentration is held constant and the pH is varied, showed that both genistein and daidzein show a sorption maximum on goethite near pH 7, with slightly lower sorption at lower pH values and steadily decreasing sorption as pH increases. With kaolinite, daidzein shows minimal sorption above pH 7 (K_D ~ 30 L/kg), with increasing sorption with decreasing pH, up to K_D ~ 70 L/kg at pH 3.5. Genistein does not sorb above pH 10, but sorption increases with decreasing pH.
Daidzein displays weakly pH-dependent sorption on montmorillonite, but at all pH values tested, higher concentrations of $CaCl_2$ increased the sorption. Increased concentration of NaCl had a weaker effect than $CaCl_2$. With Na-montmorillonite, genistein showed increasing sorption with increasing pH, while Ca-montmorillonite showed a weak pH effect, but a marked increase in sorption with the highest concentration of $CaCl_2$ (0.5 M) compared to lower concentrations (0.1 M and 0.01 M). Interpretation of the sorption edges, including modeling, are in progress and sorption experiments using organic matter-amended soil minerals as well as natural sediments are planned.

Experiments determining the biological degradation rates of genistein were conducted using water collected from the Minnesota River at Mankato, and the Okabena Creek at Brewster. Variation in the degradation rates of genistein based on concentration, temperature, source water, and season were determined. These rates are summarized below.

Degradation rate coefficients for genistein degradation when fed genistein at an initial concentration of 500 ng/L

				Zero Order Kinetics			First Order Kinetics		
Source[1]	**Water Collection Date**[2]	**Reactor #**	**Temp [°C]**[3]	**K [µg/L-hr]**	**R^2**	**Average ± 95% CI [µg/L-hr]**	**K [1/hr]**	**R^2**	**Average ± 95% CI [1/hr]**
MN River, Mankato, MN	6/6/12	1	20	7.60 E-03	0.827	$7.00 \times 10^{-3} \pm 2.42 \times 10^{-3}$	-2.98E-02	0.998	$3.39 \times 10^{-2} \pm 1.76 \times 10^{-3}$
		2		8.20 E-03	0.798		-3.60E-02	0.992	
		3		5.19 E-03	0.707		-3.76E-02	0.997	
MN River, Mankato, MN	5/14/12	1	20	3.48 E-03	0.944	$4.88 \times 10^{-3} \pm 5.64 \times 10^{-4}$	-1.83E-02	0.976	$2.24 \times 10^{-2} \pm 1.28 \times 10^{-3}$
		2		5.72×10^{-3}	0.951		-2.52E-02	0.995	
		3		5.43×10^{-3}	0.959		-2.38E-02	0.987	
MN River, Mankato, MN	6/6/12	1	10	1.86 E-03	0.786	$2.07 \times 10^{-3} \pm 6.25 \times 10^{-4}$	-2.03E-02	0.98	$1.76 \times 10^{-2} \pm 1.71 \times 10^{-3}$
		2		2.33 E-03	0.901		-1.93E-02	0.991	
		3		2.01 E-03	0.955		-1.30E-02	0.994	
Okabena Creek, Brewster, MN	6/6/12	1	20	7.17 E-03	0.929	$7.96 \times 10^{-3} \pm 1.79 \times 10^{-3}$	-1.62E-02	0.81	$2.11 \times 10^{-2} \pm 6.99 \times 10^{-3}$
		2		9.93 E-03	0.877		-2.72E-02	0.818	
		3		6.77 E-03	0.89		-2.00E-02	0.771	

[1] Source refers to either the Minnesota River at Mankato, MN or Brewster, MN where bulk water was collected and subsequently used to run batch reactors
[2] Water collection date is the date of bulk water collection
[3] Reactors were either run at 10 or 20 °C, which is indicated by the temperature column

Degradation rate coefficients for genistein degradation when fed genistein at an initial concentration of 100 µg/L

				Zero Order Kinetics			First Order Kinetics		
Source	**Water Collection Date**	**Reactor #**	**Temp [C]**	**K [µg/L-hr]**	**R^2**	**Average ± 95% CI [µg/L-hr]**	**K [1/hr]**	**R^2**	**Average ± 95% CI [1/hr]**
MN River, Mankato, MN	5/14/2012	M1	20	6.83	0.971	$6.08 \pm 7.68 \times 10^{-1}$	-2.78E-01	0.839	$2.31 \times 10^{-1} \pm 7.48 \times 10^{-2}$
		M2		5.59	0.98		-1.72E-01	0.879	
		M3		5.81	0.977		-2.43E-01	0.868	
MN River, Mankato, MN	11/8/2011	M1	20	2.32	0.964	2.86 ± 1.12	-4.99E-02	0.837	$9.37 \times 10^{-2} \pm 2.34 \times 10^{-1}$
		M2		4.21	0.935		-9.49E-02	0.798	
		M3		2.04	0.971		-5.46E-02	0.767	
Okabena Creek, Brewster, MN	11/8/2011	B1	20	2.29	0.635	2.92 ± 2.07	-2.19E-02	0.599	$1.29 \times 10^{-1} \pm 1.83 \times 10^{-1}$
		B2		3.20	0.918		-1.63E-01	0.624	
		B3		3.29	0.908		-1.75E-01	0.604	

Temperature, source water and season affected the degradation rate by as much as 100%, whereas initial genistein concentration affected rates less dramatically. High concentration experiments (100 µg/L) produced a variable lag period followed by rapid, near zero-order degradation. Half-lives varied from about 30 to 51 hours. Low concentration experiments (0.5 µg/L, or 500 ng/L) were best modeled using first order kinetics; half-lives in these experiments ranged from 18.5 to 53 hours. Overall, genistein appears to be readily degradable with highly variable rates. Our data also suggests that phytoestrogens are likely to be more persistent in cold weather months from discharges to lower-flow streams, such as Okabena Creek.

The following manuscript has been published:

Kelly MM, Arnold WA. 2012. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environmental Science and Technology*, 46(10):5396-5403.

Result Status as of January 2013

All grab samples collected upstream and downstream of the Mankato (June 2011, November 2011, and May 2012) and Brewster WWTPs (June 2012) have been analyzed. Even downstream of these suspected phytoestrogen sources, the phytoestrogens monitored in this study were only detected in the low nanogram per liter range. In Minnesota River water sampled on 6/20/11, all species, with the exception of coumestrol, were present; in the Minnesota River samples taken on 11/8/11, only one sample, the WWTP effluent from the city of Mankato, contained phytoestrogens (daidzein and formononetin). Samples from Okabena Creek and the Brewster WWTP effluent showed a similar pattern, with only genistein and daidzein detected in the low nanogram per liter range. These results again indicate that the ability to biodegrade genistein (and other phytoestrogens) is likely to be ubiquitous in the environment. This is supported by the lack of high genistein concentrations (and phytoestrogens in general) in samples downstream of probable sources.

Degradation rate coefficients for genistein under a variety of conditions are given in the July 2012 result status section. Confidence intervals were recalculated using an alternative (yet equally accepted) technique; a number of other errors were found and corrected. These are all shown as corrected with a mark-out. As mentioned above, genistein was found to degrade both readily and variably in surface water samples, with differences in degradation rates based on source water, month of sample collection, and incubation temperature.

Laboratory-scale reactor studies with a highly enriched culture were performed to determine which organism(s) were responsible for genistein degradation. Results showed that although the culture was developed under nitrifying conditions in the absence or genistein, the organisms present were able to readily degrade genistein. This supports the notion that genistein degraders could be multiple substrate utilizers. In addition, the addition of an inhibitor of nitrification, allylthiourea, halted ammonia and nitrite oxidation in the reactor, but did not stop or slow genistein degradation. This demonstrates that nitrifiers are not likely to be responsible for genistein degradation.

Therefore, the higher genistein removal rates observed by others in WWTPs that employ tertiary treatment may not be a direct result of nitrifying organisms, but rather to other microorganisms enriched under similar conditions.

The biodegradation results are currently being prepared for publication.

Experiments to determine the sorption isotherms for genistein, daidzein, and formononetin to sediment collected from the Mississippi River were performed, using well water from the SCSU lab. Formononetin had a K_D of 157, genistein had a K_D of 58, and daidzein had a K_D of 12. Isotherms for the sorption of genistein to Ca-montmorillonite were also completed at pH 6, 8.5, and 11. The K_D values were 72, 61, and 128, respectively. Isotherms for the sorption of genistein to Na-montmorillonite were completed at pH 6 and 11. The K_D values were 100 and 50, respectively.
Isotherms for the sorption of biochanin A to kaolinite, Na- and Ca-montmorillonite were also completed, showing K_D values of 1200, 82 and 1310, respectively.
Preliminary modeling efforts of the previously described sorption edges suggest that one important factor in determining the extent of sorption of genistein or daidzein to goethite is the prevalence of protonated surface sites. With respect to kaolinite sorption, although kaolinite is thought to have variable surface charge than goethite, the more important factor for sorption to kaolinite seemed to be the fraction of genistein or daidzein in the fully protonated form.

Final Report Summary:
Photolysis: UV-visible spectra for genistein and daidzein at varying pH values were taken, the pK_a values for both compounds were measured, and UV-visible spectra for each protonation state were determined. The loss of both compounds in deionized water was observed upon exposure to natural sunlight, and the quantum yields were determined for each protonation state. In Mississippi River water, direct photolysis does not account for all of the loss of genistein and daidzein. The mechanism of indirect photolysis was probed, and results suggest that daidzein is transformed mainly via direct photolysis and singlet oxygenation, while genistein is transformed mainly via reaction with triplet-state natural organic matter. These results have been incorporated into one manuscript that has been published in *Environmental Science and Technology* (included with the final report documents).

Sorption: K_d values (sorption coefficients) were obtained by sorption isotherms for genistein, daidzein, formononetin, and biochanin A on several clays and iron minerals (goethite, kaolinite, Ca- and Na-montmorillonite) and sediment collected from the Mississippi River. Biochanin A showed the highest K_d values for sorption to kaolinite and Ca-montmorillonite. Genistein exhibited the next highest K_d values for sorption to goethite at low pH values where it is mostly protonated and the goethite has protonated surface sites. Daidzein did not sorb strongly to any of the tested sorbents. When montmorillonite was the sorbent, genistein showed no obvious relationship between K_d and ion composition. For biochanin A and daidzein, however, sorption to Ca-montmorillonite was stronger than to Na-montmorillonite. Genistein and daidzein both clearly displayed pH-dependent sorption to goethite and all of the tested phytoestrogens

exhibited pH-dependent sorption to kaolinite. With respect to Mississippi River sediment, the K_d values were converted to log K_{oc} values (the sorption to organic carbon), yielding 3.88 for genistein, 3.51 for daidzein, and 4.26 for formononetin. These can therefore be considered to be moderately sorptive compounds. Compared to traditional persistent organic pollutants, isoflavones do not sorb especially strongly to clay minerals, iron oxides, or natural sediments. Nevertheless, if high concentrations or frequent discharges of isoflavones are released to natural waters, sorption to settling particles will play a role in the attenuation of these compounds. The role of sorption in the total attenuation of phytoestrogens will depend on sediment composition, as well as non-sorptive factors such as time of day and dissolved organic matter composition, which influence removal by photolysis and biological activity.

Using this data, the sorbed fraction of genistein and daidzein in a hypothetical lake were calculated. In a lake at pH 8.5 and 0.01 M NaCl with 5 mg/L of suspended sediment, composed of 50% quartz, 10% goethite, 10% kaolinite, 10% Na-montmorillonite, and 20% organic matter, 0.77% of the genistein and 0.32% of the daidzein would sorb to particles. Under the same conditions, but with 25 mg/L suspended sediment, 3.8% of the genistein and 1.6% of the daidzein introduced to the lake would sorb to particles. Assuming a typical settling velocity for suspended particles, 1.9% of the genistein and 0.8% of the daidzein would settle with particles every day. Therefore, sorption to particles may be important for aquatic systems receiving inputs of isoflavones in which the water moves very slowly. These results have been incorporated into one manuscript that will be submitted shortly (August/September 2013) for publication in the peer-reviewed literature.

Biodegradation: The persistence of genistein and daidzein in natural aquatic systems was assessed in riverine samples. Initial concentration, temperature, sample location, and time of sample collection were varied. Genistein and daidzein were found to be readily biodegradable at all tested concentrations (0.5 to 100 µg/L), at both 10 and 20°C, in samples collected in different seasons, and in samples from three different rivers. Genistein degradation appeared to be performed by heterotrophic bacteria capable of scavenging a range of low-concentration carbonaceous compounds for survival. These results suggest that there is minimal risk of the presence of high phytoestrogen concentrations in receiving waters if at least some wastewater treatment is provided at point sources. Nevertheless, caution and more research should be focused on phytoestrogen persistence at low temperatures, during which degradation rates drop and these compounds could build-up in the water column or in sediment and impact aquatic wildlife as a result. These results have been incorporated into one manuscript that has been submitted for publication in the peer-reviewed literature (included with the final report documents).

Modeling and overall assessment: With an understanding of the chemical properties and biodegradability of genistein and daidzein developed, it was possible to predict the environmental fate of these two compounds under specific environmental conditions. To do so, a box model representing a lake and a multi-box model representing a stream or river were developed (inputs given below in the table).

Table 1. Parameters used as inputs to the box model of a lake

	Summer, GEN	Winter, GEN	Summer, DDZ	Winter, DDZ
Volume (m^3)	1.50×10^8	1.50×10^8	1.50×10^8	1.50×10^8
Concentration of phytoestrogens leaving the lake (ng/L)	1.50×10^6	1.50×10^6	9.8×10^5	9.8×10^5
Flow of water entering and leaving the lake (m^3/day)	3.40×10^5	3.40×10^5	3.40×10^5	3.40×10^5
Fraction of effluent entering the lake	0.1	0.1	0.1	0.1

The models shown describe the concentration of genistein or daidzein in the lake in a scenario where a treatment plant discharging to the lake begins accepting waste from a soy-processing facility, resulting in an effluent concentration of 150,000 ng/L genistein and 98,000 ng/L daidzein at a flow rate of 3.40×10^4 m^3/day. The steady-state concentration reached for each scenario is given in the table below.

Table 2. Steady-state concentrations for genistein and daidzein in summer and winter in a model Lake

	Summer, GEN	Winter, GEN	Summer, DDZ	Winter, DDZ
No removal	1.50×10^4	1.50×10^4	9.80×10^3	9.80×10^3
Photolysis only	5.77×10^3	6.37×10^3	2.00×10^2	4.13×10^2
Biodegradation only	0	0	0	0
Sedimentation only	1.50×10^4	1.50×10^4	9.78×10^2	9.78×10^2
All processes	0	0	0	0

To evaluate the persistence of genistein and daidzein in streams, a multi-box model was constructed. Two streams were imagined, one (“Minnesota River”) with a higher flowrate (20,000 m^3/hr) and lower fraction of effluent entering the stream (0.05), and one (“Zumbro River”) with a lower flowrate (500 m^3/hr) and a higher fraction of effluent entering the stream (0.4). The volume of each box was set to assume a cross sectional area of 10 m^2, and the length of the box was set to 10 m. It was assumed that each box came to equilibrium within its residence time, Q/V. When this model was evaluated, it was seen that biodegradation alone controlled genistein and daidzein persistence and did not allow either compound to accumulate. Direct photolysis of daidzein appears to speed the rate of loss very slightly.

From these models we see quite clearly that where biodegradation of concentrated effluents is occurring, genistein and daidzein will not accumulate. Even at sites where biodegradation is very slow, direct and indirect photolysis efficiently degrade genistein and daidzein, particularly in the summer months, and could hold the concentrations in a lake below the biological threshold of 1000 ng/L. Nevertheless, because the biodegradation products of genistein and daidzein have been shown to possess androgenic or anti-estrogenic activity (see Result 2 below), aquatic organisms could still be impacted. To verify these modeling results, samples were taken downstream from two wastewater discharges; very low phytoestrogen concentrations were observed (low ng/L levels). The field sampling results have been incorporated into a manuscript that has been submitted for publication in the peer-reviewed literature (included with the final report documents).

RESULT 2: ***Determine the impact of the phytoestrogens on fathead minnows***

Description:
The fathead minnow will be used as the biological model for this research, as this organism is used as a screening organism for EDCs by the US EPA and it is an important component of the Minnesota aquatic food chain. While two previous studies have indicated behavioral and physical changes in phytoestrogen-exposed fish, these studies did not use realistic compound concentrations, mixtures, or fish native to Minnesota. We propose to assess the effects of genistein and daidzein (singularly and in mixtures) over a range of environmentally-relevant concentrations in controlled laboratory experiments. Three life stages (embryo, larva, adult) of the fathead minnow will be investigated to assess developmental, behavioral, and physical changes (including feminization) in the fish. For each life stage, fish will be exposed to three concentrations of each compound (spanning observed environmental concentrations) and to three mixtures of the two compounds. Following exposure, embryos and larvae will be assessed in their ability to perform innate predator avoidance behaviors. Adult fathead minnows (males and females) will be assessed for changes in their reproductive behavior. Fish will also be analyzed for vitellogenin concentrations (a precursor protein involved in egg production and a sign of feminization of male fish) and their livers and reproductive organs will be evaluated for changes. Finally, we will also perform in-stream experiments downstream of the discharge of the two soy-processing facilities to verify the results of laboratory experiments.

Summary Budget Information for Result 2:	**ENRTF Budget:**	**$**	**120,000**
	Amount Spent:	**$**	**120,000**
	Balance:	**$**	**0**

Deliverable/Outcome	Completion Date	Budget
1. Determine the effects of phytoestrogens on embryonic and larval fathead minnow behavior	4/30/11	49,000

2. Determine the effects of phytoestrogens on the reproductive behavior of mature fathead minnows	4/30/12	42,500
3. Determine the effects of phytoestrogens on the physiology of mature fathead minnows (feminization, liver, and reproductive organ changes)	4/30/13	28,500

Result Completion Date: April 30, 2013

Result Status as of January 2011:

To determine the biological effects range of three common phytoestrogens, we exposed groups of 20 males and 20 females each to one of three phytoestrogens (Daidzein; Genistein, Formononetin) at 1,000 ng/L or mixtures of the three compounds at a combined concentration of 1,000 ng/L or 3,000 ng/L for 21 days. An ethanol carrier control and a 50 ng/L 17ß-estradiol positive control were added for quality control purposes. Immediately following exposure, 10 males/10 females from each treatment were sacrificed for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology. The remaining ten males/females per treatment were assessed in an aggression behavioral assay and then also sacrificed and assessed as described above.

We have completed the exposure experiment, and have completed histological and vitellogenin analysis and are currently conducting statistical analysis of the collected data sets. Water samples have been forwarded to the Novak laboratory for analysis.

Result Status as of July 2011:

We have completed the analysis of the first complete exposure experiment of fathead minnows in a flow-through exposure system to a suite of phytoestrogens and their mixtures. All biological data have been gathered (including vitellogenin analysis and histopathology) and statistically analyzed. The results indicate a weak, but statistically significant induction of plasma vitellogenin in male fish exposed to daidzein at 1 μg/L and the mixture of all three phytoestrogens (daidzein, genistein, and formononetin) at 1 μg/L each (Figure 1).

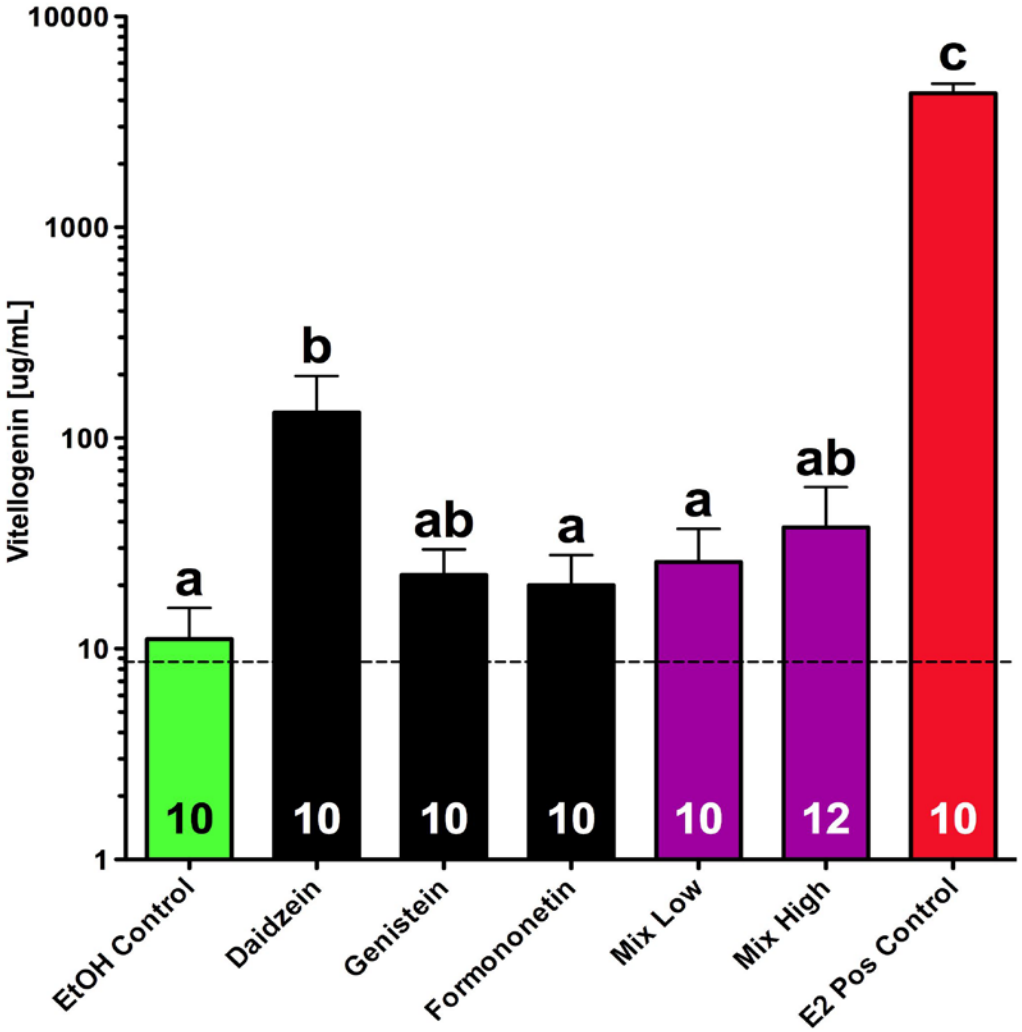


Figure. Male plasma vitellogenin concentrations (ug/mL) after 21-day flow-through exposure to phytoestrogens individually and in mixture.

Statistical analysis based on log transformed vtg data. ANOVA with Tukey's post test ($p<0.0001$). Letters indicate significant differences between treatments at $p<0.05$; numbers in each column indicate sample size. Dotted line indicates ELISA assay detection limit.

The results gathered from the first 21-day exposure study are being validated through a second experiment using additional reproductive endpoints. This exposure begun recently and will be completed by the end of August 2011.

Result Status as of January 2012:

We have completed two 21-day flow-through exposure experiments of male and female fathead minnows to three phytoestrogens at environmentally relevant concentrations (1,000 ng/L each of daidzein, genistein, formononetin). In each of these duplicate experiments we also included mixture treatments combining all three phytoestrogens at 330 ng/L each or 1,000 ng/L each. Finally, the duplicate experiments contained a

negative ethanol carrier control and a positive (50 ng/L) estradiol control. Each treatment consisted of two aquaria of 10 male fish each and two aquaria of 10 female fish each.

Immediately following exposure, we sacrificed 10 males and 10 females from each treatment for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology. The remaining ten (or less) fish per treatment were paired one male, one female and placed into the behavioral assay setup. These fish pairs (of the same previous exposure treatment) were then assessed in the aggression and reproduction assays for the following 14 days. We checked each reproductive pair daily for egg production with any deposited eggs being removed and counted to assess female fecundity. Egg viability was assessed three days after egg deposition by counting those eggs that contained eye-spots. All fertilized eggs were maintained until hatching to enumerate hatching success in each treatment.

All analysis of plasma samples for vitellogenin and liver and gonad samples for histopathology has been completed and results again indicate that feminization is occurring for phytoestrogen exposure. Data sets are currently been subjected to statistical analysis. In addition, we are developing an experimental design for a follow-up experiment to assess the effects of phytoestrogen metabolic breakdown products on mature male and female fathead minnows. We plan to conduct this experiment in spring 2012 with analysis completed by summer 2012.

Result Status as of July 2012

A third 21-day flow-through exposure experiment was conducted to compare the effects of phytoestrogens and their biological degradation products on fathead minnow anatomy, physiology, behavior and reproduction. We compared the same phytoestrogen mixture (1000 ng/L) used in the two previous flow-through exposure experiments with this same phytoestrogen mixture after biodegradation in a biologically active reactor (referred to as the “bioreactor”). The bioreactor (4 L) was modeled after a WWTP. Influent and effluent flows were 3 L/day. Media was made daily, to which phytoestrogens (500 ug/L each of formenonetin, genistein, and daidzein) were added. The bioreactor operated on a 14-day solids retention time. Aeration provided dissolved oxygen above 4 mg/L during the experiment. Samples were taken of the influent and effluent of the reactor and analyzed for pH, dissolved oxygen, ammonia, chemical oxygen demand, and suspended solids. Ten male and female baseline fish were dissected prior to exposure, with the equivalent number of males and females dissected after 21 days of exposure. Three treatments were compared: (1) the phytoestrogen mixture (positive control), (2) the bioreactor effluent containing degraded phytoestrogens, and (3) an ethanol control.

As in the two previous experiments, we sacrificed 10 males and 10 females from each treatment for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology immediately following

exposure. The remaining ten (or less) fish per treatment were paired one male, one female and placed into the behavioral assay setup. These fish pairs (of the same previous exposure treatment) were then assessed in the aggression and reproduction assays for the following 14 days. We checked each reproductive pair daily for egg production with any deposited eggs being removed and counted to assess female fecundity. Egg viability was assessed three days after egg deposition by counting those eggs that contained eye-spots. All fertilized eggs were maintained until hatching to enumerate hatching success in each treatment.

All analysis of plasma samples for vitellogenin and liver and gonad samples for histopathology has been completed and results are currently been subjected to statistical analysis.

Result Status as of January 2013

All adult replicate exposure experiments (see previous result status reporting) have been fully analyzed and suggest only subtle effects (on anatomy, physiology and behavior of mature fathead minnows) as a result of exposure to these compounds singly or in mixtures. Consequently we investigated the effects of these compounds on larval fathead minnows, a potentially more vulnerable life stage.

Three separate exposure experiments were conducted to compare larval fathead minnow escape performance, growth, and survival. The experiments were used to contrast un-degraded phytoestrogens (**compound exposure**), biodegraded phytoestrogens (presumably phytoestrogen degradation product exposure; **degraded exposure**), and phytoestrogens released from the sediment to which they were sorbed (**sediment exposure**). Larvae were exposed 21 days via 24-hour static renewal for the aqueous phytoestrogen exposures, and exchanged every 72 hours for the sediment experiment. Larval minnows (25) were randomly assigned to one of three exposure beakers providing 75 larvae per each treatment. Larval fish were fed 1 mL of live brine shrimp twice daily. Following exposure, a subset of 10 larvae from each beaker were assessed for predator avoidance performance following previously published protocols. Briefly, we transferred larvae individually to a testing arena overlaying a grid system and a vibrational stimulus. Using a high-speed camera and motioncamera software (Redlake), we recorded larval startle response. We measured body length (mm) latency (msec), velocity (BL/msec), and total escape response (BL/msec). At a later date, video files were analyzed using Image J. Remaining larvae were counted to use mortality as an endpoint. Temperature (ambient air and water), pH, and conductivity were measured daily and did not significantly differ between treatments in any experiment. Hach test strips were used to compare water chemistry every 4 days (total Cl, free Cl, total hardness, total alkalinity, and pH). No significant difference was found in any experiment.

Compound exposure. We compared six treatment groups: water blank, ethanol carrier, daidzein, formononetin, genistein, and a mixture (mixture high from earlier adult exposures). Survivors were enumerated after the escape performance assay. EtOH

survival was significantly higher than Genistein ($p<0.001$), Daidzein ($p<0.001$), and mixture exposure ($p=0.001$). No difference was found when comparing formononetin and EtOH survival ($p=0.12$).

Degraded exposure. We compared five treatment groups: degraded lake water control, degraded daidzein, degraded formononetin, degraded genistein, and degraded mixture (mixture high from earlier adult exposures). We found a significant difference in larval growth between daidzein and the mixture treatments ($p = 0.04$). Mean body length (mm) of fish exposed to the phytoestrogen mixture was lower than those exposed to daidzein. No other significance was detected.

Sediment exposure. We compared four treatment groups: sediment control, sediment daidzein, sediment formononetin, and sediment genistein. We did not see a significant difference on growth or escape performance. When comparing survival we found fish survival was higher for well water exposure relative to genistein ($p=0.002$) and formononetin ($p<0.001$). No significance was found for survival when comparing daidzein ($p=0.87$) to the well water control.

Final Report Summary:
All of the data was thoroughly analyzed in the context of controls and confirmational water chemistry data from the exposure experiments. The response of larval and sexually mature fathead minnows to environmentally relevant concentrations of three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture was quantified. In addition, organismal responses in larval and sexually mature fathead minnows were quantified following exposure to microbiologically degraded phytoestrogens (genistein, daidzein, and formononetin). Larval survival was significantly reduced in genistein, formononetin and mixture treatments while adult male fish only exhibited subtle changes to their anatomy, physiology and behavior. Daidzein-exposed adult females produced significantly greater quantities of eggs. Products of the microbiological degradation of parent phytoestrogens did not have an effect on larval survival, growth, or predator avoidance. Female adult fathead minnows exposed to these degradation products produced significantly fewer eggs than those exposed to a control, but no other morphological, physiological, or behavioral changes were observed with male or female minnows. As phytoestrogens may bind to sediment, this route of larval exposure was also investigated, especially since larval fathead minnows remain in close contact with substrate for long periods of time. Larval fathead minnow exposures to sediments sorbed with genistein, daidzein, formononetin, and their mixtures through were conducted for 21-day exposure periods. After assessing larval survival growth and behavior in detail, no statistically significant decline compared to an unexposed control sediment was observed in any of the matrices. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates. These results have been incorporated into two manuscripts that have been submitted for publication in the peer-reviewed literature (included with the final report documents) and a third manuscript

currently under co-author review.

V. TOTAL ENRTF PROJECT BUDGET:

Personnel:	**$**	**187,000**
(William Arnold and Paige Novak, paid for 4% effort; 2 graduate students for approximately 1.75 years (averaging 29% effort over 3 years))		
Contracts:	**$**	**82,000**
(Some of the work will be conducted at St. Cloud State University (Result 2). The subcontract amount will include Co-PI salary (Heiko Schoenfuss, paid for 2.5% effort (less paid effort because of summer teaching commitments)), one undergraduate research assistant ($12,000 for 3 years), supplies for experiments (fish, etc., $36,000), and half of the funds required for travel to the site for sampling and in-stream experiments ($1,000)). Graduate research assistants hired and paid through UMN will work on both Result 1 and Result 2 and an additional graduate research assistant will be hired through St. Cloud State University to work on Result 2.)		
Equipment/Tools/Supplies:	**$**	**70,000**
(Laboratory supplies, analytical costs, and instrument maintenance and repair costs)		
Acquisition, including easements:	**$**	**0**
Travel:	**$**	**1,000**
(Travel to sites for sampling)		
Additional Budget Items:	**$**	**0**
TOTAL ENRTF PROJECT BUDGET:	**$**	**340,000**

Explanation of Capital Expenditures Greater Than $3,500: *None*

VI. PROJECT STRATEGY:

A. Project Partners: Dr. Paige Novak (University of MN), an expert in the occurrence of phytoestrogens and their biological transformation, will lead the project and coordinate the research. Dr. Heiko Schoenfuss (St. Cloud State University), an expert on the impact of endocrine disrupting compounds on aquatic biota, will direct the biological impact research. Dr. William Arnold (UMN), an expert in photolysis of endocrine disrupting compounds and building kinetic models, will direct the studies on phytoestrogen fate with P. Novak. We have contacted personnel at the Mankato and Brewster wastewater facilities and have permission to sample their effluent.

B. Project Impact and Long-term Strategy: The proposed research fits into a larger research agenda centered at the University of Minnesota that is focused on the problem of environmental estrogens and endocrine disruptors in the State's surface waters. Although the proposed research will be completed in the allotted 3-year period with the requested financial resources, it complements current and prior research in this area. When taken together, the research performed or proposed by the University of Minnesota and its partners (e.g., St. Cloud State University) will provide a more

complete picture of important sources and loads of estrogens/endocrine disruptors, the fate of these compounds in both engineered and natural systems, and potential strategies (communication or engineering) to mitigate the threat caused by these compounds.

C. Other Funds Proposed to be Spent during the Project Period: The three PIs will each devote effort to the project that will be unpaid, 2% for both Novak and Arnold and 3.5% for Schoenfuss.

D. Spending History: None.

VII. DISSEMINATION:

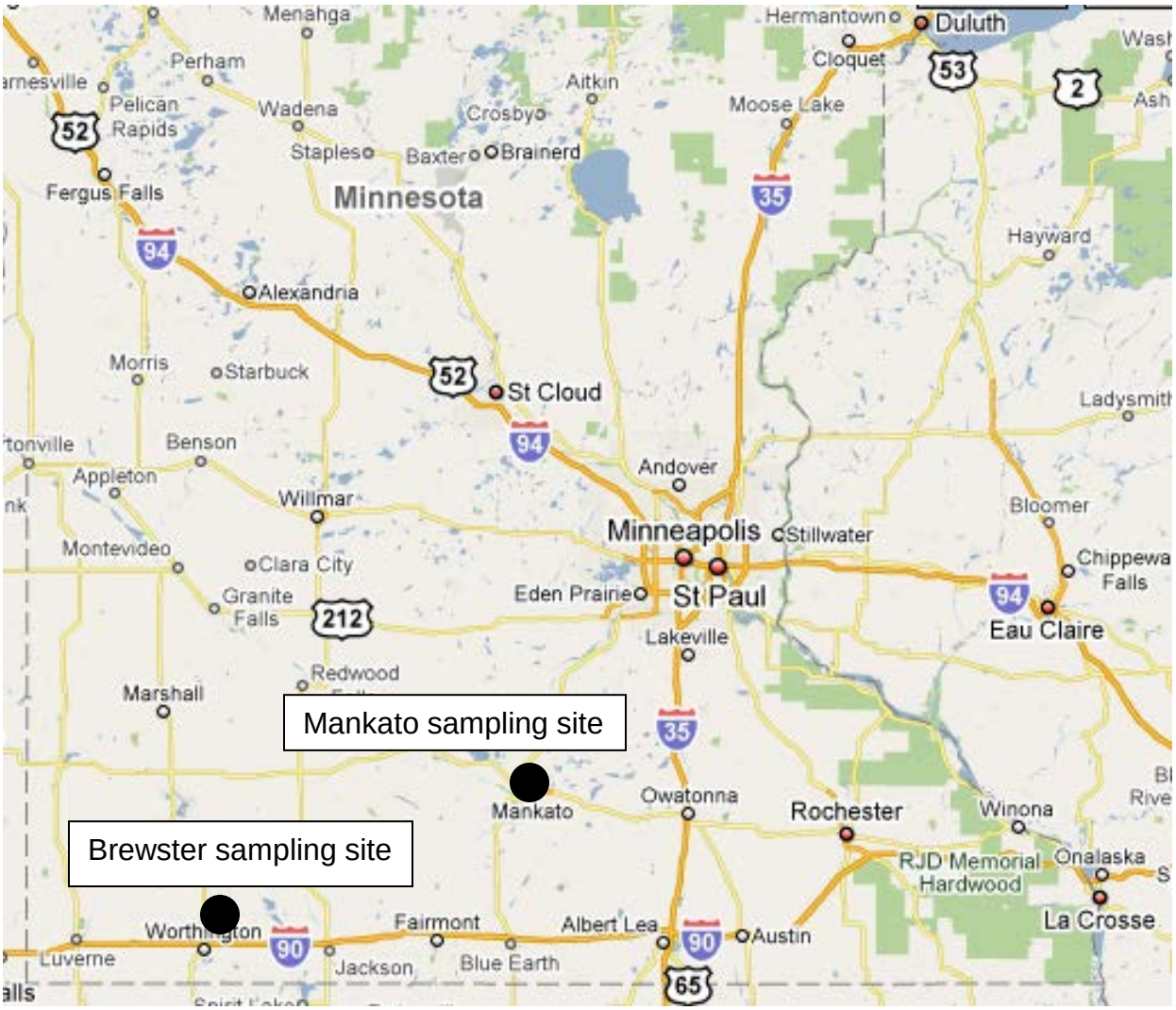
The target audience for results from this research will be professionals in the area of wastewater treatment, watershed management, and industry. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the MDA and MPCA, and environmental consultants. 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.

VIII. REPORTING REQUIREMENTS:

Periodic Work Program progress reports will be submitted not later than January 2011, July 2011, January 2012, July 2012, and January 2013. A final Work Program report and associated products will be submitted between June 30 and August 1, 2013 as requested by the LCCMR.

IX. RESEARCH PROJECTS:

See Attachment B.


Menahga
Hermantown
Duluth
Perham
Cloquet
Aitkin
Pelican Rapids
Wadena
Crosby
Moose Lake
Staples
Baxter
Brainerd
Minnesota
Fergus Falls
Hayward
Alexandria
Morris
Starbuck
St Cloud
Benson
Appleton
Andover
Willmar
Bloomer
Minneapolis
Stillwater
Montevideo
Clara City
Chippewa Falls
Granite Falls
Eden Prairie
St Paul
Eau Claire
Lakeville
Redwood
Marshall
Mankato sampling site
Mankato
Owatonna
Rochester
Winona
Brewster sampling site
RJD Memorial Hardwood
Onalaska
La Crosse
Worthington
Fairmont
Albert Lea
Austin
Luverne
Jackson
Blue Earth
52
94
35
53
2
212
90
65

Final Attachment A: Budget Detail for 2010 Projects

Project Title: Fate and ecological impacts of phytoestrogens

Project Manager Name: Paige Novak

Trust Fund Appropriation: $ *340,000*

2010 Trust Fund Budget	**Result 1 Budget:**	**Final Adjusted Result 1 Budget 8/21/2013**	**Amount Spent 06/30/2013**	**Balance 06/30/2013**	**Result 2 Budget:**	**Final Adjusted Result 2 Budget 8/21/2013**	**Amount Spent 06/13/2013**	**Balance 06/30/2013**	**TOTAL BUDGET**	**TOTAL BALANCE**
	Determine the chemical and biological fate of phytoestrogens in surface waters				Determine the impact of the phytoestrogens on fathead minnows					
BUDGET ITEM										
PERSONNEL: wages and benefits	149,000	149,000	149,000	0	~~38,000~~	40,058	40,058	0	189,058	0
Paige Novak (4%)										
William Arnold (4%)										
Graduate Research Assistant (37.5%)										
Graduate Research Assistant (37.5%)										
Subcontract (Some of the work will be conducted at St. Cloud State University (Result 2). The subcontract amount will include Co-PI salary (Heiko Schoenfuss, paid for 2.5% effort), one undergraduate research assistant ($12,000 for 3 years), supplies for experiments (fish, etc., $36,000), and half of the funds required for travel to the site for sampling and in-stream experiments ($1,000)). Graduate research assistants hired and paid through UMN will work on both Result 1 and Result 2 and an additional graduate research assistant will be hired through					82,000	82,000	82,000	0	82,000	0
Other direct operating costs										
Supplies (Laboratory supplies including, but not limited to, gas supply for the LC/MS, glassware, syringes, chemical standards, membrane filters, clean-up columns, and disposables; Analytical costs; Instrument maintenance and repair costs)	~~70,000~~	67,942	67,345	597					67,942	597
Travel expenses in Minnesota	1,000	1,000	682	318					1,000	318
COLUMN TOTAL	**~~$220,000~~**	**$217,942**	**$217,027**	**$915**	**~~$120,000~~**	**$122,058**	**$122,058**	**$0**	**$340,000**	**$915**

Running Head: biodegradation of phytoestrogens and biological effects

***Corresponding author:** Paige Novak
University of Minnesota
122 Civil Engineering Building
500 Pillsbury Drive S.E.
Minneapolis, MN 55455, USA
(USA) 612-626-9846 (phone)
(USA) 612-626-7750 (fax)
novak010@umn.edu

Total number of words in text, references, tables and figure legends: **5283**

PHYTOESTROGENS IN THE ENVIRONMENT: II. MICROBIOLOGICAL DEGRADATION OF PHYTOESTROGENS AND THE RESPONSE OF FATHEAD MINNOWS TO DEGRADATE EXPOSURE

MEGAN M. KELLY†, NATHAN T. FLEISCHHACKER‡, DANIEL C. REARICK§, WILLIAM A. ARNOLD†‡, HEIKO L. SCHOENFUSS§, AND PAIGE J. NOVAK†‡*

† Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

Abstract

Phytoestrogens are endocrine active compounds derived from plants, including the isoflavones genistein and daidzein. These compounds have been detected at the µg/L level in the effluents of plant-processing industries and municipal treatment plants, and at the ng/L level in surface water around the world. The persistence of genistein and daidzein in natural aquatic systems was assessed in riverine samples. Initial concentration, temperature, sample location, and time of sample collection were varied. Genistein and daidzein were found to be readily biodegradable at all tested concentrations, at both 10 and 20°C, in samples collected at different seasons, and in samples from three different rivers. In addition, organismal responses in larval and sexually mature fathead minnows (*Pimephales promelas*) were quantified following exposure to microbiologically degraded phytoestrogens (genistein, daidzein, and formononetin). Products of the microbiological degradation of parent phytoestrogens did not have an effect on larval survival, growth, or predator avoidance. Female adult fathead minnows exposed to these degradation products produced significantly fewer eggs than those exposed to a control, but no other morphological, physiological, or behavioral changes were observed with male or female minnows. This research suggests that although phytoestrogens are not likely to be persistent in aquatic systems, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates.

Keywords: endocrine-active compounds, reproduction, behavior, phytoestrogens, biodegradation

INTRODUCTION

Cultivated and uncultivated plants contain varying concentrations of phytoestrogens, a class of plant-produced endocrine-active compounds. Genistein, daidzein and their methylated derivatives, biochanin A and formononetin, respectively, are the primary isoflavones found in many legumes [1-4]. These compounds are also produced in the highest concentrations from cultivated plants and tend to be the most estrogenic in this compound class [1-9]. Indeed, numerous studies have linked phytoestrogen exposure in fish to wide ranging reproductive [10-15], developmental [10,12,14], and behavioral effects [10-11,13] at levels as low as 1 µg/L [16].

Of particular concern is the presence of phytoestrogens in industrial and municipal wastewater effluents and in non-point source agricultural runoff. In a study of wastewater effluents from nineteen industries, eight contained high phytoestrogen concentrations, dominated by genistein and daidzein: biodiesel refinery effluent (1.3-22.5 µg/L), ethanol production effluent (4.7 µg/L), and effluents from soy milk (250 µg/L), soy oil (127 µg/L), dairy (39.9 µg/L), barbeque meat (30.8 µg/L), and peanut processing (6.3 µg/L) [17]. Effluents from pulp and paper mills have also been found to contain high concentrations of phytoestrogens [*e.g.*, 18], with one study measuring genistein concentrations in pulp and paper mill effluent at 10.1 µg/L [19]. In addition to industrial processes, humans are capable of excreting up to several milligrams of phytoestrogens per day depending on diet, which points to the likely presence of these compounds in municipal wastewater treatment plant (WWTP) effluents [20]. Studies conducted on municipal WWTP effluents have detected the presence of phytoestrogens at a range of concentrations (<1 to 1,380 ng/L) [17,21-23]. Agricultural field runoff, from

land-applied livestock manure and decomposing crop vegetation, can also act as a non-point source of phytoestrogens into the environment [6,24-26].

Although the details of phytoestrogen degradation within receiving waters have yet to be examined, degradation across municipal WWTPs has been observed [17,21,25]. The microorganisms responsible are unknown. In addition, the products of degradation have not been identified or assessed for biological activity. If phytoestrogen degradation products are discharged from point sources, such as industrial or municipal WWTPs, and phytoestrogens present in surface water are also degraded, the biological significance of this process should be assessed to gain a holistic understanding of the impacts of this compound class on aquatic organisms. If estrogenicity decreases following degradation, as has been observed with the microbiological degradation of steroidal estrogens [*e.g.*, 27], the implementation of strategies to control phytoestrogen discharge and impact should be straightforward (*i.e.*, wastewater treatment via microbiological degradation).

Given the rapid expansion of plant processing for fuel and dietary products in the United States, more must be learned about how phytoestrogens biodegrade and the effect of those degradates once discharged into the environment. With this in mind, three major objectives were evaluated during this study. First, the biodegradation rates of two common phytoestrogens, genistein and daidzein, were determined over a range of concentrations in surface water. Second, the biodegradation of the model phytoestrogen genistein was further explored as a function of incubation temperature, surface water source, and time of surface water collection. Genistein biodegradation was also assessed in the presence of an inhibitor of nitrification. Finally, the responses

of larval and sexually mature fathead minnows (*Pimephales promelas*) were quantified following exposure to phytoestrogen degradates. Larvae were exposed to the degradates of the commonly detected phytoestrogens genistein and daidzein, and to the degradates of a mixture of genistein, daidzein, and formononetin. Adults were exposed to the degradation products of the mixture of genistein, daidzein, and formononetin. Mixtures were investigated to assess the expected presence of phytoestrogen/phytoestrogen degradate mixtures in discharges [*e.g.*, 17,26]. Results from these three objectives will facilitate for a more inclusive assessment of phytoestrogen risk to fish based on genistein and daidzein persistence in a variety of surface waters and the biological effects of their degradates.

METHODS

Water collection and experimental setup

Water was collected on multiple dates from the Minnesota River (November 8, 2011, May 14, 2012, June 6, 2012) and Okabena Creek (November 8, 2011, June 6, 2012) from the top 0.5-m of the water column, approximately 1 m from the riverbank and approximately 500 m downstream from the Mankato, MN and Brewster, MN WWTP outfalls, respectively. Water at this location was past the mixing zone and represented the mixed river water as opposed to the treatment plant effluent. Surface water from the Mississippi River was collected in the same fashion, at the East River Flats (Minneapolis, MN), on June 26, 2013. Samples were collected in 23-L carboys, packed on ice, transported to the laboratory, and stored at 4°C until use. Water samples were used in experiments within two weeks of collection.

Genistein and daidzein biodegradation kinetics in Mississippi River water. Triplicate batch reactors were constructed in 160-mL serum bottles with gas permeable caps. The bottles were autoclaved for 30 minutes and a methanol stock solution of genistein or daidzein was added to reach an initial genistein or daidzein concentration of 50, 10, 1, or 0.5 µg/L. The methanol was allowed to evaporate overnight. The bottles were then filled with 120 mL Mississippi River water. Control bottles were constructed at an initial concentration of 1 µg/L by adding 50 mM sodium azide. Bottles were sacrificed over time, concentrated by solid phase extraction (SPE) followed by rotary evaporation, and analyzed by high-pressure liquid chromatography (HPLC). All four concentrations of genistein were run simultaneously, separate from all four concentrations of daidzein. Estradiol was added as a surrogate to the samples at a concentration of 10 µg/L before SPE.

At an initial concentration of 100 µg/L, triplicate reactors for genistein and daidzein were constructed in the same manner, but with only 40 mL of river water. The reactors were subsampled with a syringe. The samples were filtered through a 0.2 µm PTFE syringe filter (Restek, Bellefonte, PA) into HPLC vials and analyzed by HPLC without concentration. Three control reactors containing both genistein and daidzein and 50 mM sodium azide were constructed in the same manner as the experimental reactors.

Genistein biodegradation under different environmental conditions. Batch reactors were setup in triplicate in sterilized 4-L Erlenmeyer flasks capped with gas permeable sponge stoppers. Reactors were covered with foil to prevent genistein loss via photolysis [28]. Oxygen was supplied via stirring and headspace entrainment. Water

144 (4 L) collected from the Minnesota River or Okabena Creek was allowed to equilibrate
145 to the desired temperature (20 or 10°C), after which it was added to the reactors.
146 Genistein was added to the reactors to begin the experiments in one of two ways,
147 depending on the desired final concentration: to reach an initial concentration of 0.5
148 µg/L genistein, an aqueous stock solution was used (800 µg/L, pH 11); to reach an
149 initial concentration of 100 µg/L genistein, a methanol stock solution (100 µg/mL) was
150 added as described for the Mississippi River experiments. Initial genistein
151 concentrations were: Minnesota River water collected on May 14, 2012 and June 6,
152 2012: 0.5 µg/L genistein, collected on November 8, 2012 and May 14, 2012: 100 µg/L
153 genistein; Okabena Creek water collected on June 6, 2012: 0.5 µg/L genistein, collected
154 on November 8, 2011: 100 µg/L genistein. Negative controls amended with 50 mM
155 sodium azide were setup in triplicate as well.
156 Samples were withdrawn from the reactors over time. In those reactors receiving
157 0.5 µg/L genistein, approximately 200-mL samples were withdrawn at each sampling
158 point, amended with d_3-genistein (surrogate, Cambridge Isotopes, Andover, MA),
159 concentrated via SPE, and analyzed by liquid chromatography-mass spectrometry (LC-
160 MS). In the reactors receiving 100 µg/L genistein containing the water collected on
161 November 8, 2012, 100-mL samples were withdrawn at each sampling point, amended
162 with d_3-genistein, concentrated via SPE, and analyzed by HPLC. In the reactors
163 receiving 100 µg/L genistein containing the water collected on May 14, 2012, 0.5-mL
164 samples were withdrawn over time, syringe-filtered with a glass fiber filter, amended
165 with the d_3-genistein surrogate, and analyzed directly by LC-MS. Periodic reactor
166 samples were taken for optical density (OD, a measure of biomass growth) and

dissolved organic carbon (DOC) determination.

Genistein biodegradation under nitrifying conditions. Activated sludge was collected from the Metropolitan WWTP located in St. Paul, Minnesota. A 2.5-L continuous flow reactor was seeded with 2 mL of the collected sludge and enriched with a nitrifying media (Supporting Information, Table S1) over the course of 3 months. During enrichment, a pH of 7.5-8.5 was maintained and dissolved oxygen (DO) was maintained above 4 mg/L through the use of an air-stone. The reactor was operated with a 15-day solids retention time (SRT) and a 12-hour hydraulic residence time (HRT). The biomass from this reactor was rinsed and then used to inoculate six additional 4-L Erlenmeyer flasks at a reactor biomass concentration of approximately 50 mg/L. The initial pH of each flask was adjusted to 8 and maintained between 7.5 and 8 over the course of the experiment. Flasks were periodically amended with a concentrated $(NH_4)_2SO_4$ solution to maintain total ammonium/ammonia concentrations between 10 and 100 mg/L. To begin the experiment, genistein was added to each flask (using a 2.5 μM aqueous genistein solution) to attain a nominal concentration of 2 μg/L. The ammonia monooxygenase inhibitor, allylthiourea (80 μM), was added to flasks 22 hours after the experiment had started to stop nitrification. Triplicate killed controls (50 mM sodium azide) were run concurrently to distinguish biological removal of genistein from abiotic genistein removal. Samples (100-mL) were withdrawn, filtered through glass fiber filters (GFF, Whatman Ltd, Piscataway, NJ), amended with d_3-genistein, concentrated via SPE, and analyzed by LC-MS. Total ammonium/ammonia, nitrate, and nitrite, and suspended solids (SS) were also measured periodically.

190 *Analytical methods*

191 *Sample preparation and phytoestrogen analysis.* Sample preparation was
192 performed via SPE as described elsewhere [16]. The HPLC and LC-MS analytical
193 methods are described in detail in Rearick et al. [16]. The limits of quantification (LOQ)
194 for genistein, daidzein, and estradiol on the HPLC were 19 µg/L, 50 µg/L, and 8 µg/L,
195 respectively. For the LC-MS method the LOQ was 4.43 µg/L for genistein, 3.53 µg/L for
196 daidzein and 2.79 µg/L for formononetin. OD, SS, volatile suspended solids (VSS), and
197 DOC were also analyzed as described in the Supporting Information. Total
198 ammonium/ammonia concentration was determined on GFF-filtered samples using a
199 Thermo Scientific Orion Ammonia Specific Electrode (Waltham, MA) according to the
200 manufacturer's instructions. Nitrate and nitrite concentrations were determined by ion
201 chromatography as described in the Supporting Information.

202 *Laboratory exposure experiments*

203 The effects of microbiologically degraded phytoestrogens on larval and adult
204 fathead minnows were assessed. The potency of the microbiological products of two
205 single phytoestrogens (larvae only), genistein and daidzein, (TCI America, Portland,
206 OR, 96% and 95%, respectively), and their mixture with formononetin (Acros Organics,
207 Geel, Belgium, 99%) (larvae and adults) was assessed. After exposure, the larval
208 fathead minnows were evaluated for survival, growth and predator avoidance
209 performance and adult minnows were evaluated for reproductive impacts as a result of
210 morphological, physiological and behavioral changes, as described by Rearick et al.
211 [16].

Larval fathead minnow exposures. Products of phytoestrogen biodegradation were generated by incubating surface water (from East Lake Vadnais, Vadnais Heights, MN) with genistein or daidzein stock solutions (singly and in mixture with formononetin) for 68 hours until the parent compounds were degraded. Briefly, four solvent-rinsed 2-L Erlenmeyer flasks with Teflon coated stirbars and air-permeable stoppers were used as reactor vessels. Reactors 1 and 2 were amended with 1 mL of genistein or daidzein stock solutions (100 µg/mL in methanol), reactor 3 was amended with 1 mL of each stock solution plus 1 mL of a formononetin stock solution (100 µg/mL in methanol); reactor 4 did not receive parent phytoestrogens. The methanol was allowed to evaporate in a laminar flow cabinet after which 1 L of surface water was added to each reactor. Stirbars were set to vigorously mix and aerate reactors over a 68-hour degradation period. After 68 hours, the pH of each reactor was lowered to 2.5 using a 10 M H_2SO_4 solution. The unfiltered reactor contents were passed through SPE cartridges at a flow rate no greater than 10 mL/min. Following extraction, one column volume of Milli-Q water was passed through each SPE cartridge to remove polar salts and cartridges remained under-vacuum until all water was removed. Cartridges were then eluted with 100 mL of methanol into a 250 mL round bottom flask. The extracts underwent roto-evaporation to dryness and were reconstituted in 5 mL HPLC grade ethanol for use in the larval exposure experiments. In the larval exposure experiments, four treatment groups (degraded daidzein, degraded genistein, degraded mixture of daidzein, genistein, and formononetin (to mirror the exposure of the adult minnows), and a lake water control incubated similarly to the degraded phytoestrogen samples)

were investigated in the manner described by Rearick et al. [16]. Confirmatory water chemistry is provided in Table S2.

Adult fathead minnow exposure to phytoestrogen degradation products. Adult fathead minnows were exposed to one of two treatments: an ethanol control or the effluent from an aerobic biological reactor (described below) degrading a mixture of daidzein, genistein, and formononetin, diluted 1 to 400 to reach what would have been approximately 1,250 ng/L each if no degradation had taken place. Exposure followed a 21-day exposure regime, after which adult minnows were assessed for secondary sex characteristics (males only), hepatic-somatic index, gonadal-somatic index, vitellogenin concentration, egg production (females only), and nest defense (males only) as described by Rearick et al. [16].

To generate the phytoestrogen degradation products for the adult exposure experiment, 4-L biological reactor was seeded with activated sludge collected from the Metropolitan WWTP. The reactor was operated as a continuous-flow reactor with an HRT of 34 hours and an SRT of 14 days to ensure that the parent phytoestrogens would be fully degraded. The reactor was fed synthetic sewage media [29] modified by the removal of allylthiourea and the addition of 500 µg/L each of genistein, daidzein and formononetin in an EtOH carrier (0.4 mL EtOH per L of media). Aeration maintained the DO above 4 mg/L. The reactor operated to steady state over 35 days with a series of five sampling events during the experiment to measure pH, DO, ammonia, chemical oxygen demand, and SS (data not shown). Five water samples were also collected to verify via LC-MS that parent phytoestrogens were completely degraded before entering

the dilution chamber at the aquaria interface where the bioreactor effluent was diluted 1 to 400 with well water (Table S3).

Statistical analysis

Stata 10.1 (StataCorp, College Station, TX) was used to perform principal component analysis (PCA), Spearman's rho correlation, and construct correlation matrices. To model lag and decay of genistein and daidzein, the Gompertz curve was used:

$$C(t) = C_0 e^{be^{ct}}$$

where *C(t)* is the concentration at time *t*, C_0 is the initial phytoestrogen concentration, *b* sets the *x* displacement of the curve, and *c* sets the degradation rate. The model was fit to the Mississippi River data using Scientist for Windows (v2.1, Micromath), and to the Okabena Creek and Minnesota River data by a least-squares approach using Microsoft Excel Solver. Microsoft Excel Paired Student t-test (two-sample assuming unequal variance) was performed on genistein degradation data to assess statistical significance. Data from the fathead minnow exposure experiments were analyzed as described by Rearick et al. [16].

RESULTS

Daidzein and genistein degradation in surface water samples

Daidzein and genistein degradation rates in Mississippi River water as a function of initial concentration are shown in Figure 1. Degradation was rapid after a variable lag period (Supporting Information, Figures S1-S3) and although the rate increased with concentration, it did not level off at the higher initial concentrations tested. This suggests

that 100 µg/L was well below the half-saturation coefficient for both compounds and that rapid first-order degradation can be expected at likely environmental concentrations. The Gompertz model provided the best fit of the data for most of the experiments (Table 1). Data were also fit to zero- and first-order models, which fit the lowest two initial concentrations of genistein (0.5 and 1.0 µg/L) better than the Gompertz model (Table 1), perhaps simply because of the scatter in the data and the larger number of parameters to be fit by the Gompertz model.

Genistein degradation as a function of environmental conditions

Rates of biodegradation depended on initial concentration in all of the water sources examined (Table 1) and also depended on temperature and time of surface water collection (i.e., season of collection, Table 1). As anticipated, biodegradation rates decreased with decreasing incubation temperature, with a 10°C decrease in incubation temperature causing a statistically significant ($p=0.0069$) decrease of approximately 50% in the genistein first-order degradation rate coefficient in Minnesota River samples (collected June 6, 2012, 0.5 µg/L initial concentration) (Table 1). Even though the 95% confidence intervals appear high, t-tests revealed that first-order genistein degradation rate coefficients depended on the source of the surface water (Okabena Creek versus the Minnesota River, $p=0.033$) and the season of water collection (samples collected June 6, 2012 as compared to May 14, 2012, $p=0.020$) at low initial concentrations (0.5 µg/L). At an initial concentration of 100 µg/L, the first-order rate coefficients of genistein degradation were only statistically different with ≈91% confidence (Okabena Creek versus the Minnesota River, $p=0.077$; samples collected May 14, 2012 as compared to November 8, 2011, $p=0.087$) (Table 1). Genistein degradation in Mississippi River water

is shown in Figure 2. Although rate coefficients calculated for Mississippi River water were not statistically compared to those obtained with Minnesota River or Okabena Creek water as a result of the different experimental setups used, it appears that genistein was degraded at a rate of the same magnitude. Therefore, it appears that with the same water source (Minnesota River), faster genistein degradation occurred in water samples collected during warmer, and presumably more microbially active months. In addition, although different surface water samples all showed very similar rates of genistein degradation (Figure 2), subtle, yet significant differences in degradation rate did exist.

Overall abiotic losses of genistein were low (Figure 2C, Figures S1-3). No significant increase in biomass concentration as measured by OD or VSS was found in any of the experiments (data not shown).

The importance of nitrifying bacteria in the degradation of steroidal estrogens has been previously demonstrated [*e.g.*, 30]. If nitrifying bacteria are also responsible for genistein biodegradation, thoughtful treatment systems can be designed, particularly for industrial wastes in which ammonia concentrations may be lower. Thus, an experiment was performed to test if the microorganisms responsible for genistein degradation were nitrifiers. Figure 3 shows genistein degradation in a reactor containing a highly enriched nitrifying community. Genistein was degraded without lag upon addition to the reactor, despite the fact that the culture had not been exposed to either genistein or other carbon sources during a 3-month enrichment period. Upon the addition of allylthiourea, an inhibitor of nitrification, ammonia and nitrite oxidation stopped (Figure S4), but genistein degradation was unaffected (Figure 3). These results support the notion that

genistein degraders are likely to be heterotrophs able to thrive on a variety of low-concentration carbon sources produced during microbial growth, but that they are not nitrifiers. No statistically significant growth in biomass, as measured by SS, was observed during the approximately 50-hour experiment (data not shown).

Minnow Exposure to Phytoestrogen Degradates

After a 21-day exposure of fathead minnow larvae to microbiologically degraded phytoestrogens (genistein and daidzein singly, and in a mixture with formononetin), no effects on survival, escape velocity, or total escape response were observed in comparison to a lake water control control (Figures S5-6). The confirmatory water chemistry did reveal the presence of a compound at the daidzein retention time on the HPLC, either daidzein itself or a co-eluting compound produced in this biologically active system (including the lake water control) (Table S2). Because no effect was seen on larval minnows with daidzein exposure in another similar study [16], this was deemed to be unimportant. The other phytoestrogens were detected sporadically at levels ≤10% of the quantity of parent phytoestrogens originally biodegraded (taking into account dilution in the larval experiments).

Likewise, when adult minnows were exposed to the degradates of a mixture of genistein, daidzein, and formononetin, no statistically significant impacts on vitellogenin induction, liver vacuolization or gonad maturity were observed (Figure S7). Body condition factor, hepatosomatic index and gonadosomatic index also did not differ among treatments (Figure S8), nor did secondary sex characteristics and nest defense behavior (Figure S9). In contrast to the other measured outcomes in this experiment, however, egg production was dramatically reduced ($p=0.0003$) in adult female minnows

exposed to phytoestrogen degradates as compared to the ethanol carrier control (Figure 4).

DISCUSSION

Genistein and daidzein are rapidly degraded at a range of concentrations and under a variety of environmental conditions, such as decreased temperature, seasons characterized by low temperature and low microbiological activity, and location (Figures 1 and 2). This degradation is apparently performed by heterotrophic bacteria capable of scavenging a range of low-concentration carbonaceous compounds for survival. Yet, some industrial effluents with concentrations as high as 151,000 ng/L genistein and 98,000 ng/L daidzein have been observed [17]. A threshold limit of 1,000 ng/L below which there is no effect on aquatic wildlife has been suggested [17], which is in agreement with recent observations [16]. Assuming zero-order kinetics and a rate of 8,950 ng genistein/(L×hr) (Table 1), an effluent containing 151,000 ng/L genistein would be reduced to 1,000 ng/L in just 17 hours. At a zero-order rate of 6,490 ng/L, an effluent containing 98,000 ng/L daidzein would be reduced to 1,000 ng/L in 15 hours. These results suggest that there is minimal risk of the presence of high phytoestrogen concentrations in receiving waters if at least some wastewater treatment is provided at point sources. Nevertheless, caution and more research should be focused on phytoestrogen persistence at low temperatures, during which degradation rates drop (Table 1) and these compounds could build-up in the water column or in sediment and impact aquatic wildlife as a result [16].

Although biodegradation is expected to decrease the exposure of aquatic wildlife to genistein and daidzein within hours, the products of degradation are not known. The exposure of adult female minnows to a mixture of genistein, daidzein, and formononetin degradates resulted in significantly less egg production; other biological endpoints in adult male and female minnows and in larval minnows were unaffected upon exposure to microbiologically degraded genistein, daidzein, and formononetin. It seems clear that while the estrogenic activity of the parent compounds was eliminated via microbiological degradation, one or more components in the degradate mixture exhibited an androgenic and/or antiestrogenic effect in adult minnows. Pulp and paper mill effluent, which has been shown to contain genistein [19], has also been shown to have similar androgenic effects on both mosquitofish [31,32] and goldfish [31]. It is possible that a degradation product or products of the phytoestrogens in the mill effluent is responsible for these results. Future research should therefore also be focused on identifying the degradation products of phytoestrogens and their mode of action in fish.

ACKNOWLEDGMENTS

Funding for this project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR). We thank the undergraduate research students at St. Cloud State University who assisted in the fish dissections.

REFERENCES

1. Franke A, Custer L, Cerna C. 1994. Quantitation of phytoestrogens in legumes by HPLC. *J. Agr. Food Chem.* 42:1905–1913.
2. Kuhnle G, Dell'Aquila C, Low Y. 2007. Extraction and quantification of phytoestrogens in foods using automated solid-phase extraction and LC/MS/MS. *Anal. Chem.* 23:9234-9.
3. Liggins J, Bluck L, Runswick S, Atkinson C. 2000. Daidzein and genistein content of fruits and nuts. *J. Nutr. Biochem.* 11:326-31.
4. Liggins J, Mulligan A, Runswick S. 2002. Daidzein and genistein content of cereals. *Eur. J. Clin. Nutr.* 10:961-6.
5. Gutendorf B, Westendorf J. 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* 166:79-89.
6. Hartmann N, Erbs M, Wettstein F. 2008. Environmental exposure to estrogenic and other myco-and phytotoxins. *Chimia.* 62:364-7.
7. Kiparissis Y, Balch G, Metcalfe T. 2003. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka Oryzias latipes. *Environ. Health Persp.* 111:1158-63.
8. Kuiper G, Lemmen J, Carlsson B, Corton J. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* 139:4252-63.

9. Latonnelle K, Fostier A, Le Menn F. 2002. Binding affinities of hepatic nuclear estrogen receptors for phytoestrogens in rainbow trout (Oncorhynchus mykiss) and Siberian sturgeon (Acipenser baeri) *Gen. Comp. Endocr*. 129:69-79.
10. Clotfelter E, Rodriguez A. 2006. Behavioral changes in fish exposed to phytoestrogens. *Environ. Poll.* 144:833-9.
11. Jefferson W, Padilla-Banks E. 2007. Disruption of the developing female reproductive system by phytoestrogens: genistein as an example. *Mol. Nutr. Food Res.* 51:832-844.
12. MacLatchy D, Van der Kraak G. 1995. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharm.* 134:305-12.
13. Pelissero C, Le Menn F, Kaushick S. 1991. Estrogenic effect of dietary soya bean meal on vitellogenesis in cultured siberian sturgeon Acipenser baeri. *Gen. Comp. Endocr.* 83:447-57.
14. Tremblay L, Van der Kraak G. 1999. Comparison between the effects of the phytosterol β-Sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. *Environ. Toxicol. Chem.* 18:329-336.
15. Hewitt LM, Kovacs TG, Dube MG, MacLatchy DL, Martel PH, McMaster ME, Paice MG, Parrott JL, van den Heuvel MR, Van Der Kraak GJ. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: roles of individual compounds and mill operating conditions. *Environ Toxicol Chem* 3:682-697.
16. Rearick DC, Fleischhacker NT, Kelly MM, Arnold, WA, Novak PJ, Schoenfuss HL. Environmental occurrence of phytoestrogens and exposure effects on fathead minnows. *Environ. Toxicol. Chem.* submitted.

17. Lundgren M, Novak P. 2009. Quantification of phytoestrogens in industrial waste streams. *Environ. Toxicol. Chem.* 28:2318-23.
18. Mahmood-Khan Z, Hall E. 2003. Occurrence and removal of plant sterols in pulp and paper mill effluents. *J. Environ. Eng. Sci.* 2:17-26.
19. Kiparissis Y, Hughes R, Metcalfe C. 2001. Identification of the isoflavonoid genistein in bleached kraft mill effluent. *Environ. Sci. Tech.* 35:2423-7.
20. Low Y, Taylor J, Grace P, Dowsett M. 2005. Polymorphisms in the CYP19 gene may affect the positive correlations between serum and urine phytoestrogen metabolites and plasma androgen concentrations in men. *J. Nutr*. 135:2680-2686.
21. Bacaloni A, Cavaliere C, Faberi A, Foglia P. 2005. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* 531:229-237.
22. Kang J, Price W. 2009. Occurrence of phytoestrogens in municipal wastewater and surface waters. *J. Environ. Monitor.* 11:1477-83.
23. Laganà A, Bacaloni A, de Leva I, Faberi A, Fago G. 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Anal. Chim. Acta* 501:79-88.
24. Burnison B, Hartmann A, Lister A. 2003. A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. *Environ. Toxicol. Chem.* 22:2243-50.
25. Hoerger C, Wettstein F. 2009. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ. Sci. Technol.* 43:6151-7.

459 26. Kolpin D, Hoerger C, Meyer M. 2010. Phytoestrogens and mycotoxins in Iowa
460 streams: an examination of underinvestigated compounds in agricultural basins. *J.*
461 *Environ. Qual.* 39:2089-99.
462 27. Muller M, Rabenoelina F, Balaguer P. 2008. Chemical and biological analysis of
463 endocrine-disrupting hormones and estrogenic activity in an advanced sewage
464 treatment plant. *Environ. Toxicol. Chem.* 27:1649-58.
465 28. Kelly M, Arnold W. 2012. Direct and Indirect Photolysis of the Phytoestrogens
466 Genistein and Daidzein. *Environ. Sci. Technol.* 46:5396-403.
467 29. Novak JT, Chon DH, Curtis BA, Doyle M. 2007. Biological solids reduction using
468 the Cannibal Process. *Wat. Environ. Res.* 79:2380-2386.
469 30. Shi J, Fujisawa S, Nakai S, Hosomi M. 2004. Biodegradation of natural and
470 synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium
471 Nitrosomonas europaea. *Water Res.* 38:2322-9.
472 31. Ellis R, van den Heuvel M, Bandelj E. 2003. In vivo and in vitro assessment of the
473 androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.*
474 22:1448-56.
475 32. Orlando E, Bass D, Caltabiano L, Davis W, Gray Jr. L, Guillette Jr. L. 2007. Altered
476 development and reproduction in mosquitofish exposed to pulp and paper mill
477 effluent in the Fenholloway River, Florida USA. *Aquat. Toxicol.* 84:399-405.
478

LIST OF FIGURES

Table 1. Fitting parameters describing degradation of genistein and daidzein

	Gompertz C	First-order k	Zero-order k
Genistein			
C_0	c (h^{-1})	K (h^{-1})	K (μg $L^{-1}hr^{-1}$)
100[A]	0.36 ± 0.06	0.092 ± 0.053	8.95 ± 0.91
100[B]	0.36 ± 0.43	0.231 ± 0.075	6.08 ± 0.77
100[C]	0.54 ± 0.33	0.094 ± 0.234	2.86 ± 1.12
100[D]	0.35 ± 0.16	0.129 ± 0.183	2.92 ± 2.07
50[A]	0.39 ± 0.01	0.080 ± 0.037	2.82 ± 1.5
10[A]	0.22 ± 0.01	0.071 ± 0.019	0.271 ± 0.048
1[A]	0.28 ± 0.06	0.036 ± 0.015	0.015 ± 0.007
0.5[A]	0.26 ± 1.74	0.013 ± 0.019	0.004 ± 0.006
0.5[B]		0.022 ± 0.001	0.005 ± 0.001
0.5[E]		0.021 ± 0.007	0.008 ± 0.002
0.5[F]		0.018 ± 0.002	0.002 ± 0.001
0.5[G]		0.034 ± 0.018	0.007 ± 0.002
Daidzein			
100[A]	0.26 ± 0.01	8.71 ± 1.35	6.49 ± 0.97
50[A]	0.076 ± 0.047	2.22 ± 0.81	0.814 ± 0.164
10[A]	0.082 ± 0.038	2.11 ± 0.66	0.139 ± 0.024
1[A]	0.038 ± 0.026	1.32 ± 0.44	0.010 ± 0.002
0.5[A]	0.072 ± 0.006	1.33 ± 0.77	0.005 ± 0.002

[A]Mississippi River, June 26, 2013, 20°C

[B]Minnesota River, May 14, 2012, 20°C

[C]Minnesota River, November 8, 2011, 20°C

[D]Okabena Creek, November 8, 2011, 20°C

[E]Okabena Creek, June 6, 2012, 20°C

[F]Minnesota River, June 6, 2012, 10°C

[G]Minnesota River, June 6, 2012, 20°C

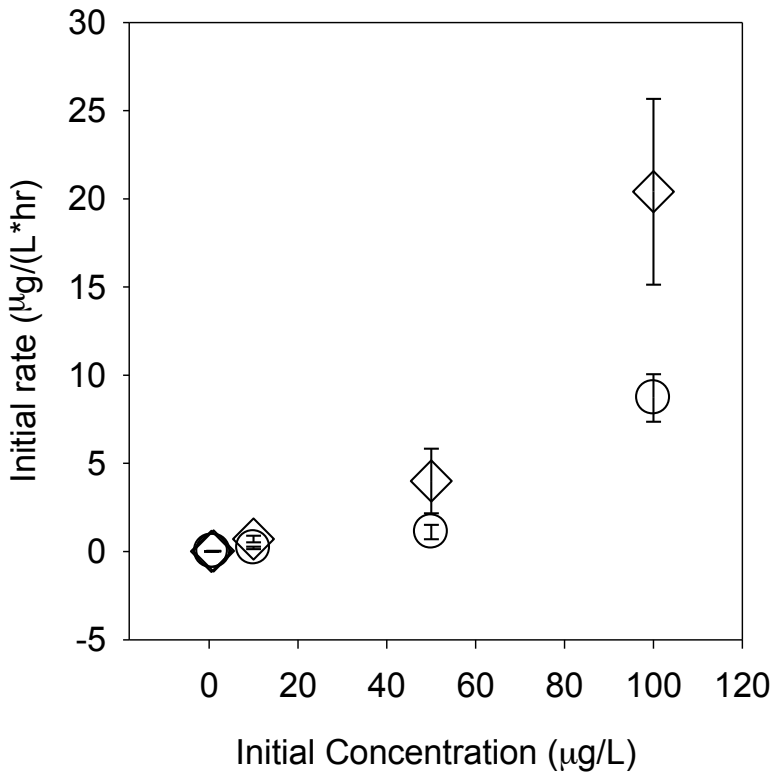


Figure 1. Initial degradation rates of genistein (diamonds) and daidzein (circles) (given as the first order rate constant multiplied by initial concentration) at different initial concentrations in Mississippi River water.

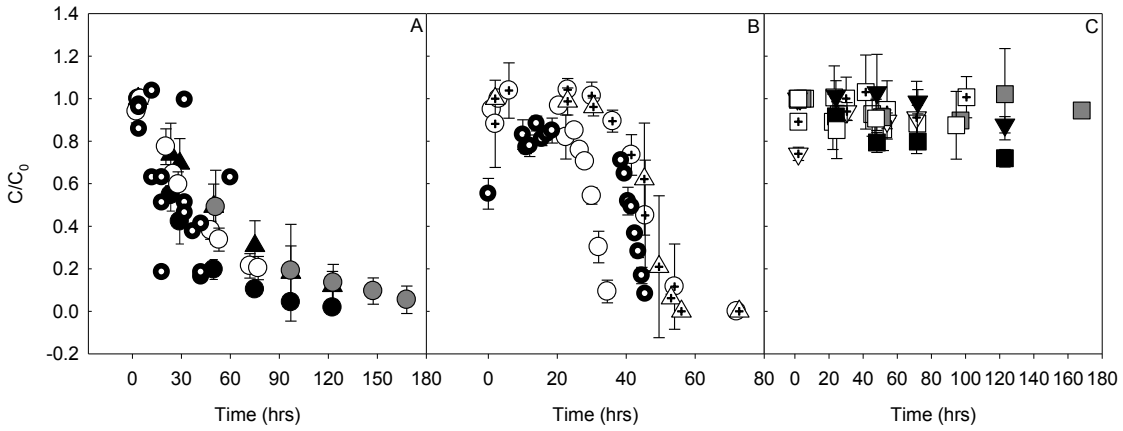


Figure 2. Degradation of genistein at an initial concentration of 0.5 µg/L (A) or 100 µg/L (B) in water collected on November 8, 2011 (cross-hairs), May 14, 2012 (white), June 6, 2012 (black), and June 26, 2013 (rings) from the Mississippi River (rings experimental, controls in SI) Okabena Creek (up triangles experimental, down triangles controls) or the Minnesota River (circles experimental, squares control) The grey points represents samples from June 6, 2012 incubated at 10°C instead of 20°C. Panel C shows controls for all experiments.

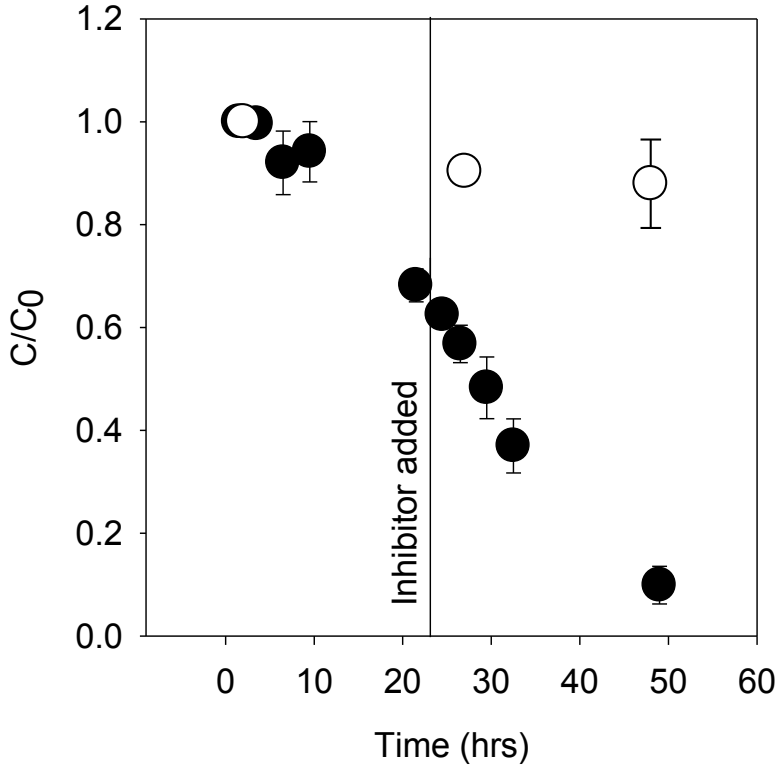


Figure 3. Degradation of genistein in an enriched nitrifying culture (black) and a killed control of the same culture (white) before and after the addition of allylthiourea, an inhibitor of ammonia oxidation.

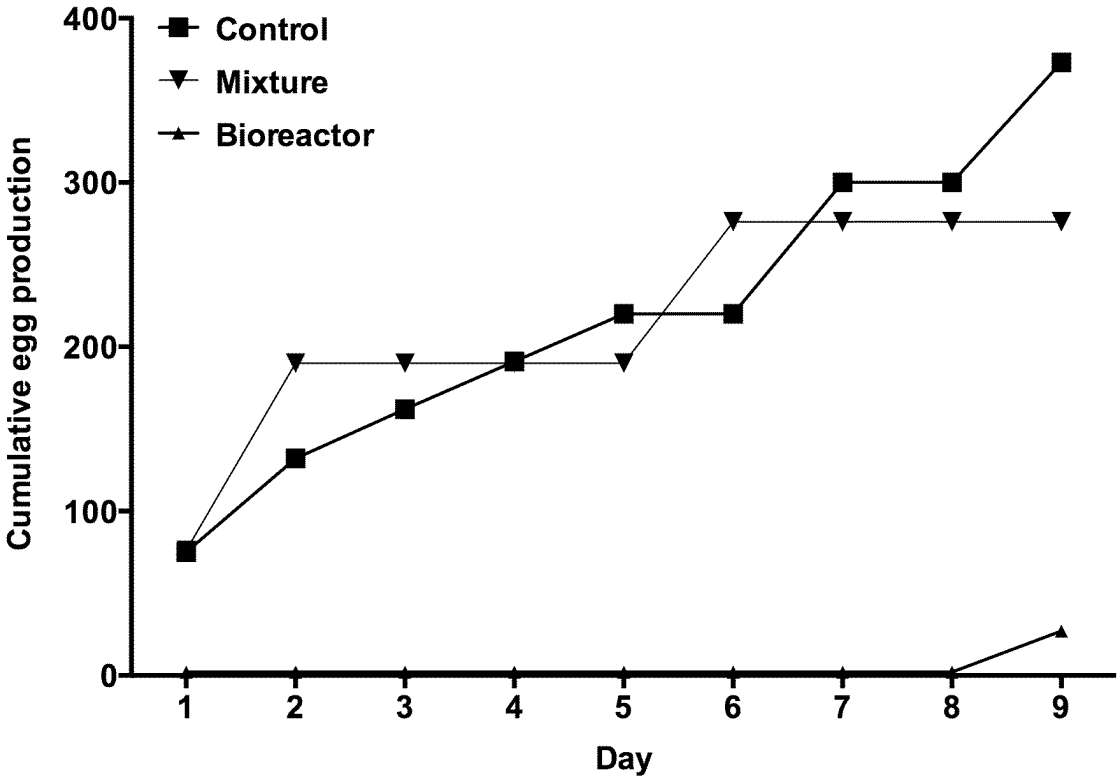


Figure 4. Cumulative egg production per treatment montiored for nine consecutive days.

SUPPORTING INFORMATION

For

PHYTOESTROGENS IN THE ENVIRONMENT: II. MICROBIOLOGICAL DEGRADATION OF PHYTOESTROGENS AND THE RESPONSE OF FATHEAD MINNOWS TO DEGRADATE EXPOSURE

MEGAN M. KELLY†, NATHAN T. FLEISCHHACKER‡, DANIEL C. REARICK§, WILLIAM A. ARNOLD†‡, HEIKO L. SCHOENFUSS§, AND PAIGE J. NOVAK†‡*

† Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

METHODS

Table S1. Nitrification reactor media

Chemical	Formula	Concentration mg/L
Sodium Phosphate	Na_2HPO_4-$7H_2O$	3,000
Potassium Phosphate	KH_2PO_4	83.3
Magnesium Sulphate	$MgSO_4$-$7H_20$	80
Calcium Chloride	$CaCl_2$	75
Sodium Bicarbonate	$NaHCO_3$	1.5
Ferric Chloride	$FeCl_3$-$6H_2O$	0.8
Copper Sulphate	$CuSO_4$	0.2
EDTA	Na_3EDTA-$4H_2O$	1
Cobalt Chloride	$CoCl_2$-$6H_2O$	2.0×10^{-4}
Zinc Sulphate	$ZnSO_4$-$7H_2O$	0.1
Sodium Molybdate	Na_2MoO_4-$2H_2O$	0.1
Manganese Chloride	$MnCl_2$-$2H_2O$	2
Ammonium Sulphate	$(NH_4)_2SO_4$	1,000

Table S2. Confirmatory water chemistry for the larval minnow phytoestrogen exposure experiment (mean ± st. err.; n=2)

Treatment	Genistein	Daidzein	Formononetin
Lake Water Control	Non-detect	40 ± 57 ng/L	25 ± 35 ng/L
Degraded genistein	30 ± 42 ng/L	115 ± 163 ng/L	Non-detect
Degraded daidzein	Non-detect	140 ± 198 ng/L	30 ± 42 ng/L
Degraded mixture	70 ± 0 ng/L	90 ± 127 ng/L	Non-detect

Note: the daidzein detection in all the treatments was thought to be a co-eluting compound as a result of biological activity in the system. A similar compound was detected in similar studies fed parent (non-degraded) phytoestrogens [S1]. In addition, similar studies showed that daidzein exposure did not cause a measureable response larval minnows [S1]; therefore, the presence of either daidzein (unlikely) or a co-eluting compound in these samples (likely) was deemed to be unimportant with respect to larval response.

Table S3. Concentrations of phytoestrogens measured (mean ± standard deviation; n=5) in the ethanol blank, the bioreactor feed, and in the bioreactor effluent in the experiment to test the effect of adult minnow exposure to the biodegraded phytoestrogens. Note: the ethanol blank and the bioreactor effluent were both further diluted 1 to 588,000 and 1 to 400, respectively, with well water prior to minnow exposure.

Treatment	Genistein	Daidzein	Formononetin
Ethanol blank	17.6 ± 2.4 ng/L	Non-detect	8.8 ± 0.7 ng/L
Bioreactor feed	108,810 ± 8,210 ng/L	65,260 ± 5,640 ng/L	163,050 ± 6,620 ng/L
Bioreactor effluent	34.3 ± 33.6 ng/L	Non-detect	8.5 ± 0.8 ng/L

Optical density (OD), suspended solids (SS), volatile suspended solids (VSS), and dissolved organic carbon (DOC) analysis. OD was measured using a Beckman DU 530 UV/VIS Spectrophotometer (Fullerton, CA) at a wavelength of 600 nm. Well-mixed samples (2 mL) were placed in cuvettes (Life Sciences, Foster City, CA) and measured three times; the average value was recorded. SS and VSS were measured according to Standard Method 2540D and 2540E [S2], respectively. Samples (20 mL) were analyzed for DOC by filtering them through a GFF, acidifying the filtrate to pH 2 with 5 M H_2SO_4, purging inorganic carbon with N_2 gas, then analyzing the residual carbon (assumed to be organic) with a Sievers 900 Portable TOC Analyzer (General Electric, Fairfield, CT).

Ion analysis. Nitrate and nitrite concentrations were determined using a Metrohm (Riverview, FL, USA) 761 ion chromatograph using a Metrohm 766 sample processor and IC Net software. The eluent solution consisted of 1mM $NaHCO_3$ and 32 mM Na_2CO_3. Regenerant was a 0.2 mM sulfuric acid solution. A combined external calibration curve for nitrate and nitrite in Milli-Q was used to quantify nitrate and nitrite.

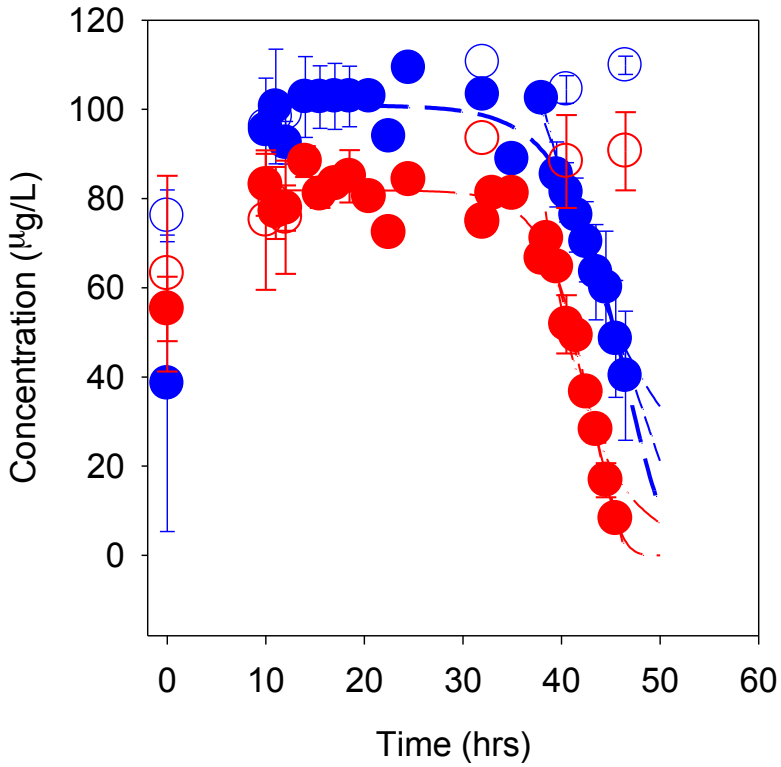


Figure S1. Degradation of genistein (red) and daidzein (blue) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

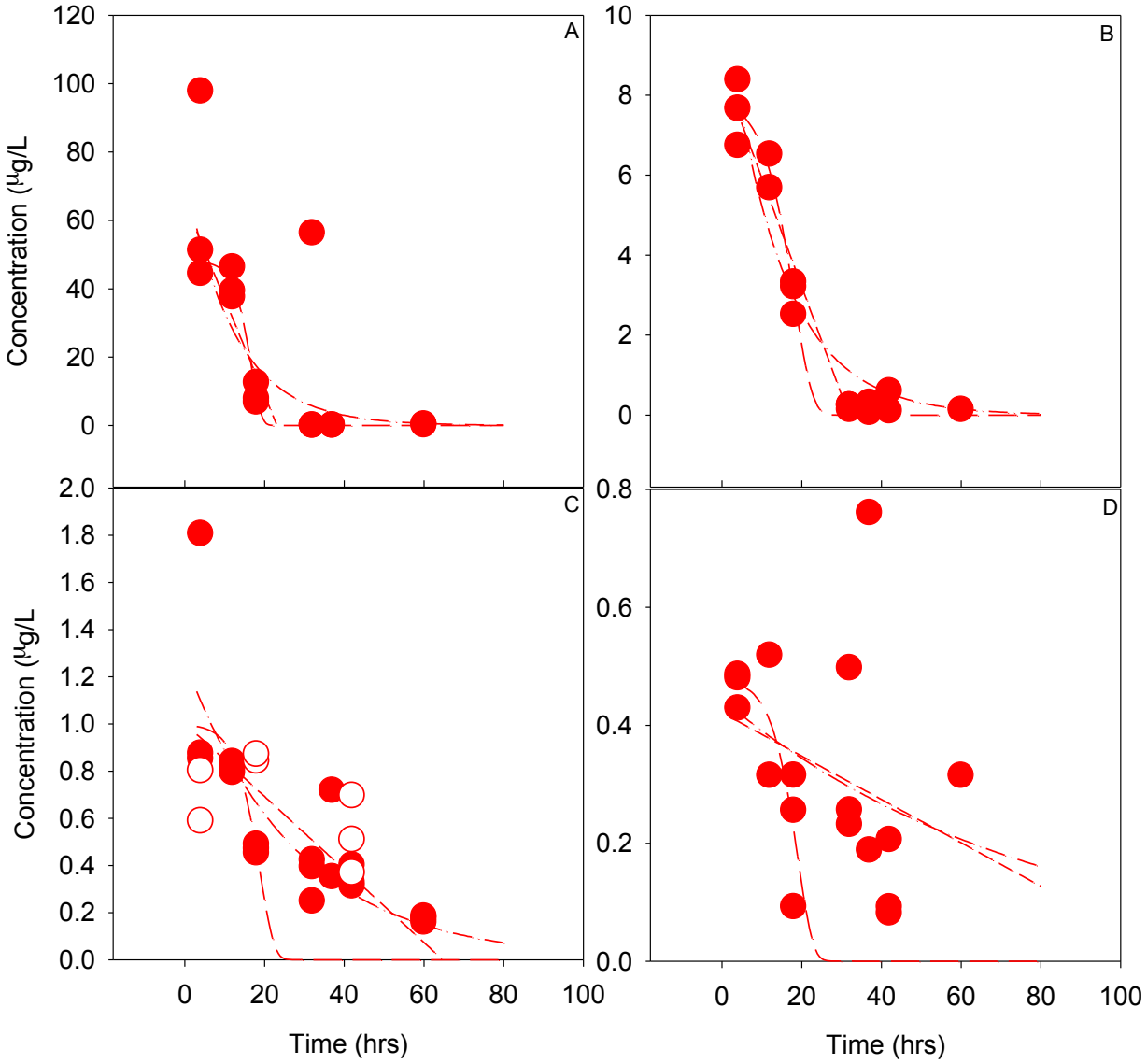


Figure S2. Degradation of genistein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

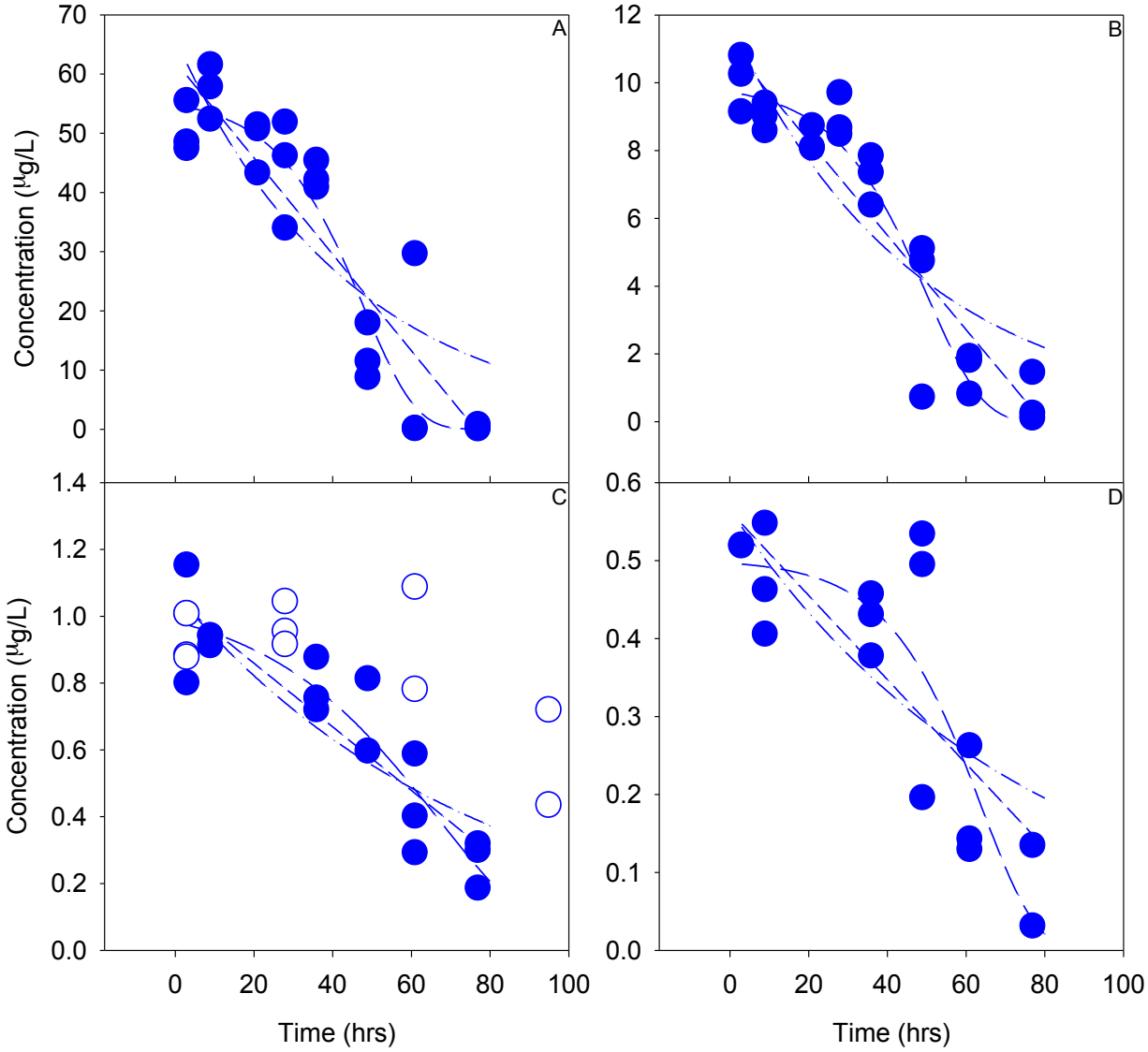


Figure S3. Degradation of daidzein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

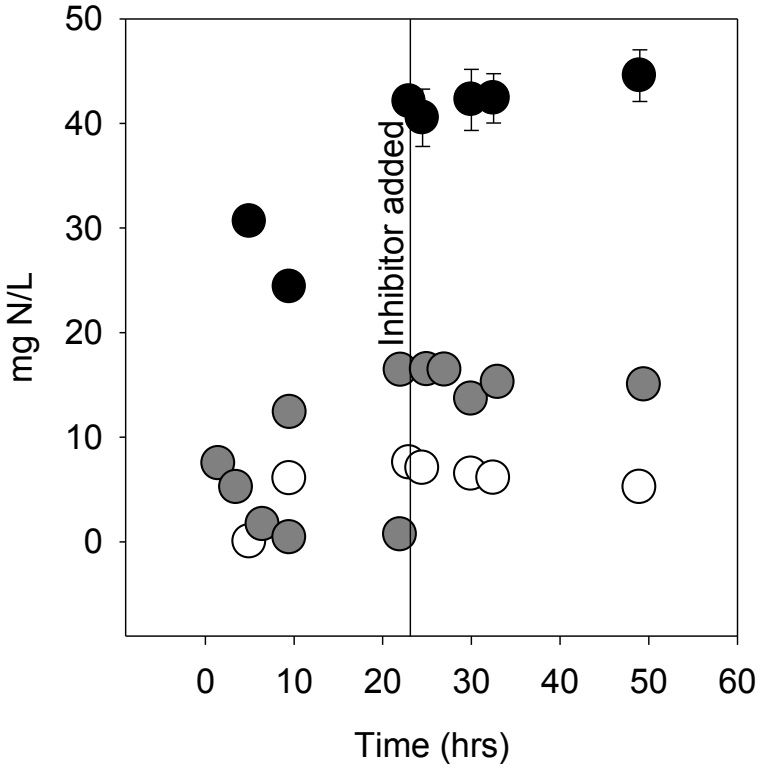


Figure S4. Concentrations of nitrite (black), nitrate (white), and ammonia (grey) in an enriched culture of nitrifying organisms before and after the addition of allylthiourea.

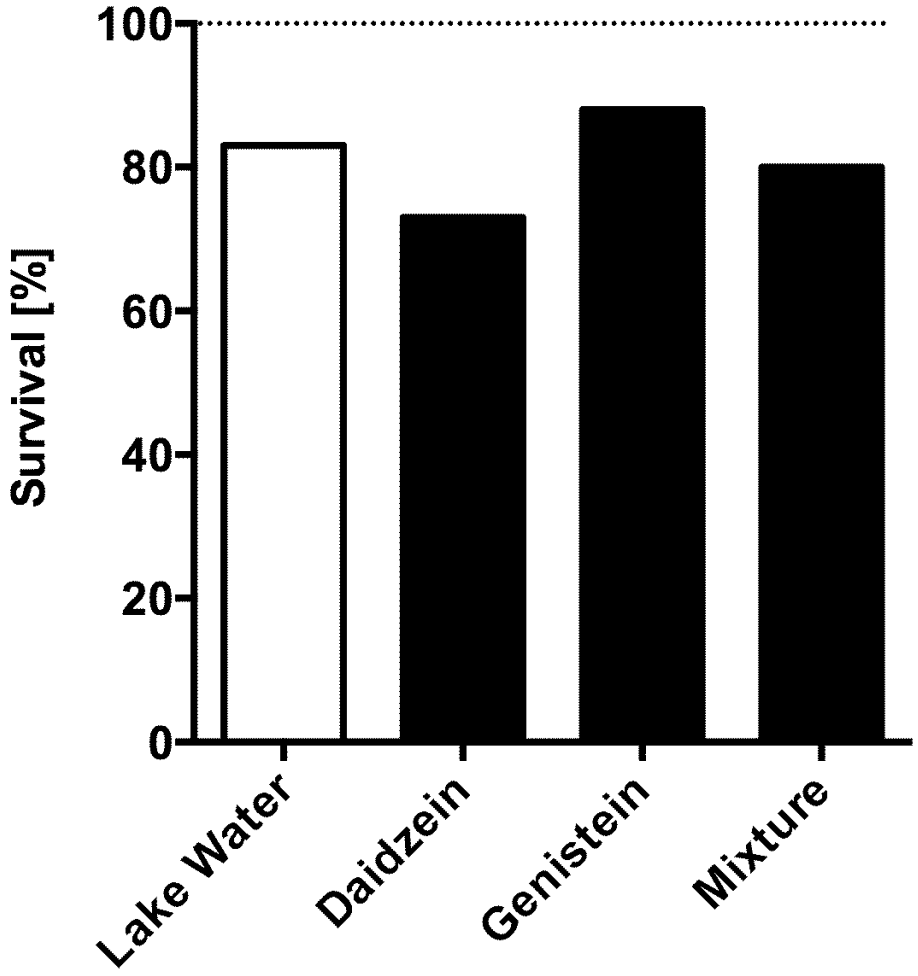


Figure S5. Percent larval fathead minnow survival during a 21-day exposure to the degradation compounds singly and in mixture. Initial cohorts were established using 75 larvae per treatment. Survival represents number of individuals remaining after behavioral testing.

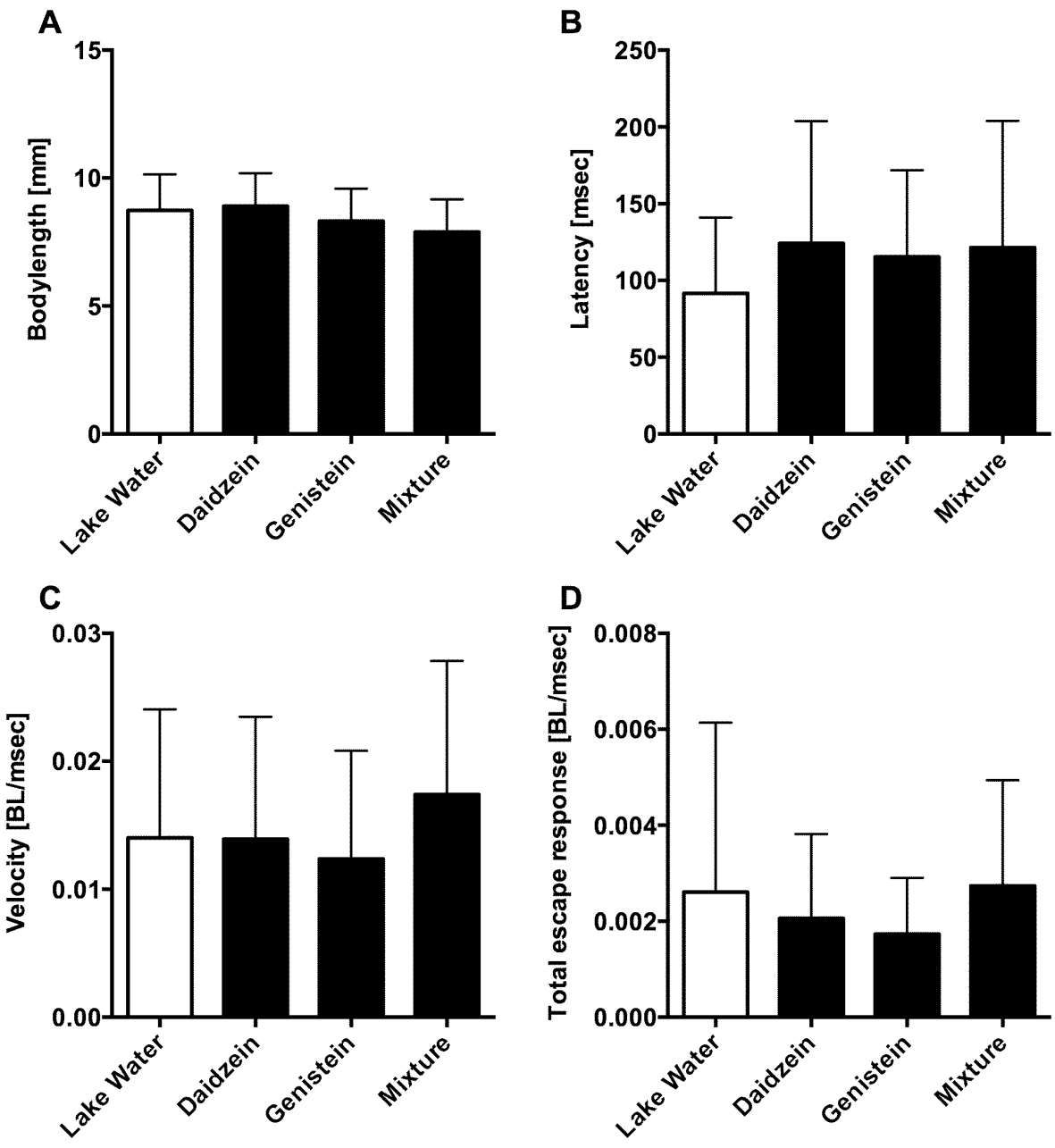


Figure S6. Larval fathead minnow escape performance following 21-day exposure to degraded phytoestrogen compounds. C-start escape performance was quantified using. (A) body length (mm); (B) mean latency (ms) from stimulus to response; (C) mean escape velocity relative to body length (BL/ms); (D) mean total escape performance (BL/ms) defined as (distance travelled/BL)/(40+latency).

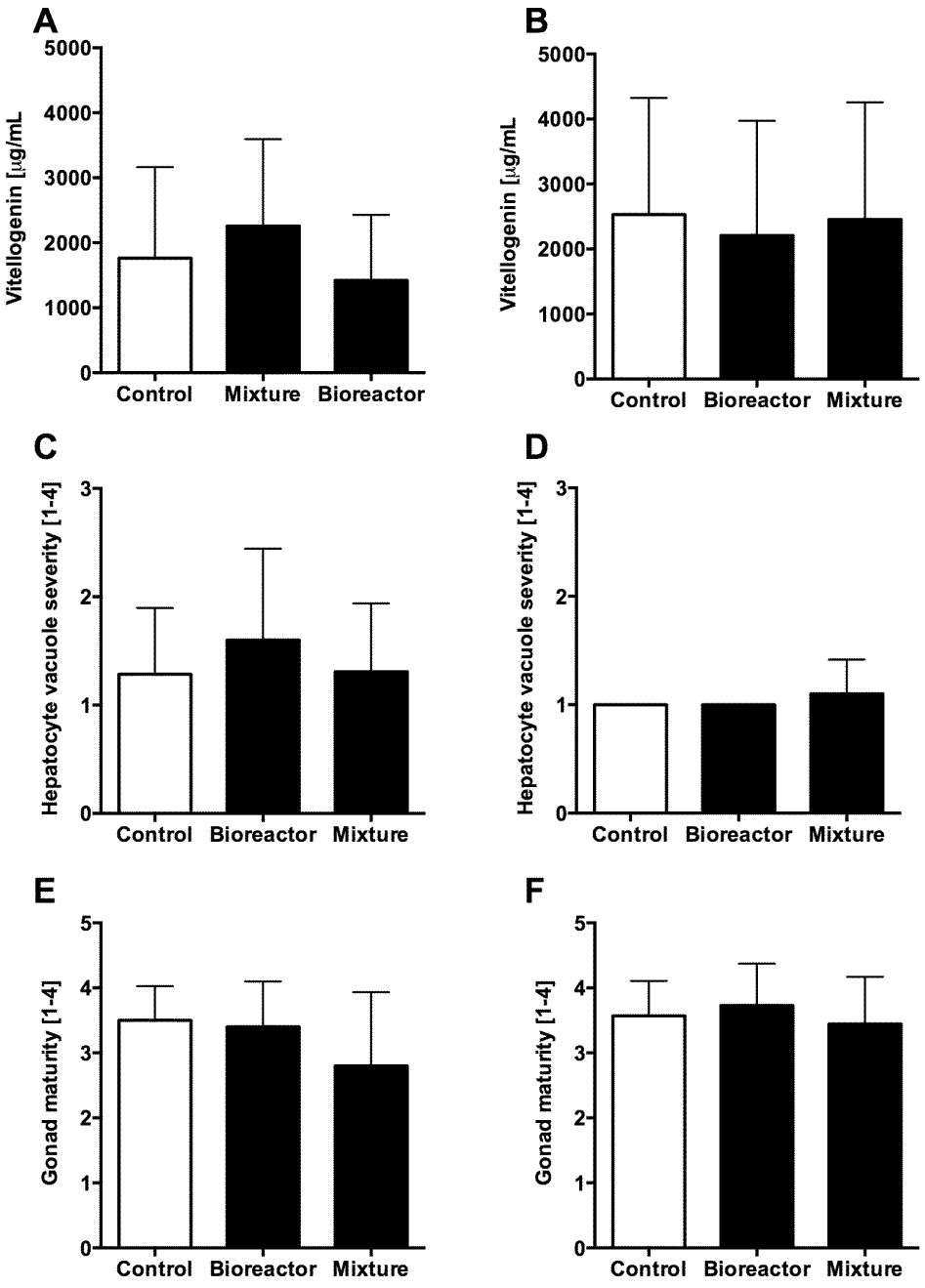


Figure S7. Mean ± standard error plasma vitellogenin concentrations (µg/mL) in male (A) and female (B) fathead minnows exposed for 21- days to the degraded phytoestrogen mixture (Daidzein, Genistein, and Formononetin 1,000 ng/L per compound). Severity of hepatocyte vacuole presence for male (C) and female (D) minnows. Testis (E) and ovarian

(F) maturity. Sample size for each treatment is listed in each column (ANOVA with Tukey's post-test).

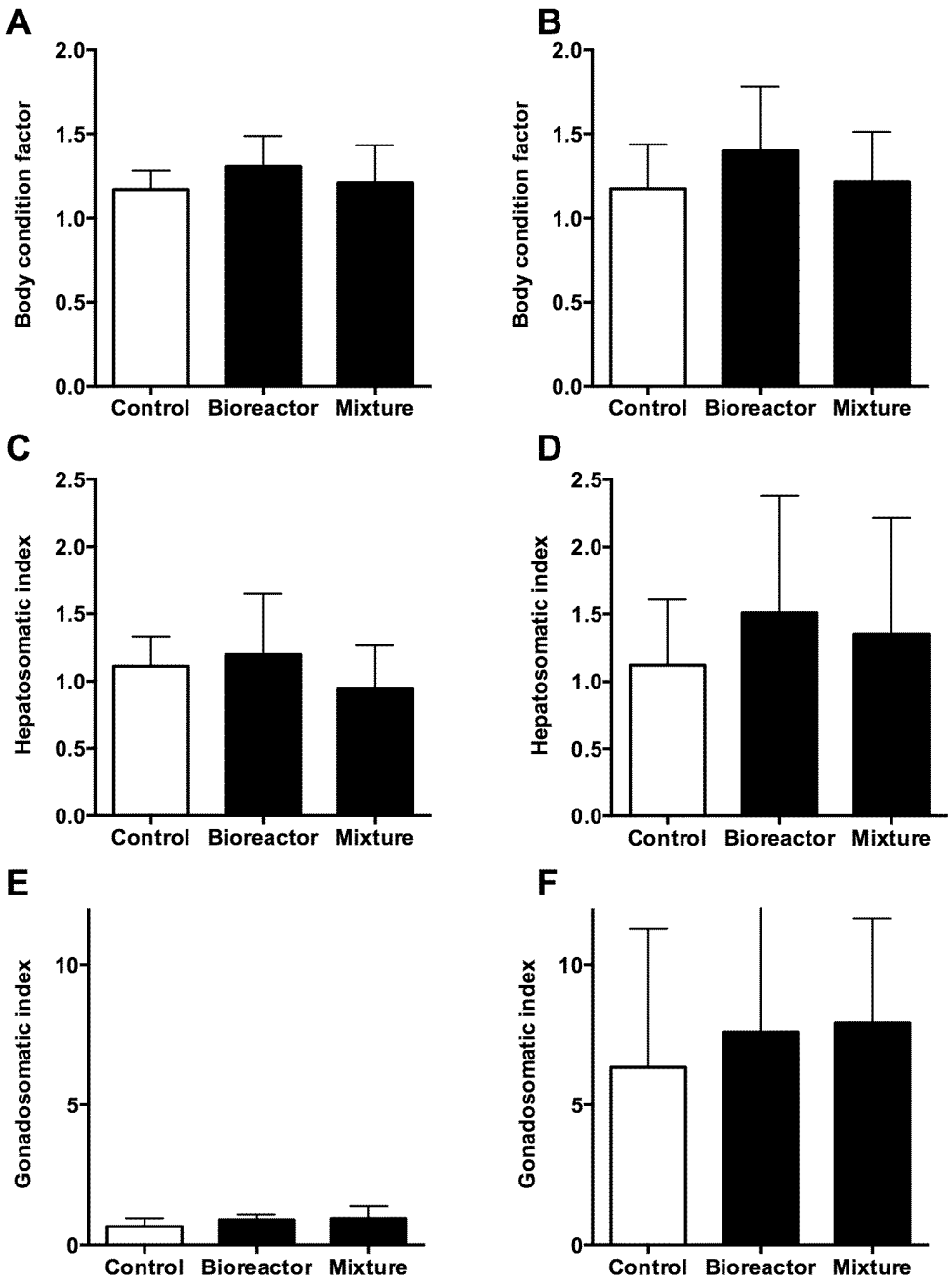


Figure S8. Body condition factor (A-male; B-female), hepatosomatic idnex (C,D) and gonadosomatic index (E,F) for fathead minnows exposed for 21 days to the degradation mixture.

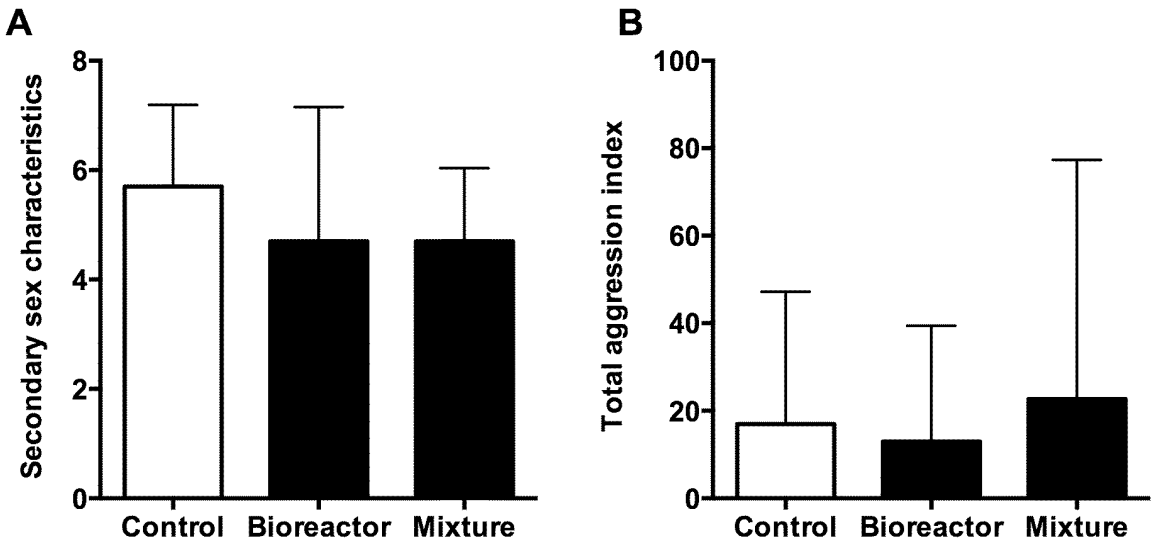


Figure S9. Mean ± standard error expression of secondary sex characteristics (A) and total aggression index (B) for male fathead minnows exposed 21-days to the degradation mixture.

REFERENCES

S1. Rearick DC, Fleischhacker NT, Kelly MM, Arnold, WA, Novak PJ, Schoenfuss HL. Environmental occurrence of phytoestrogens and exposure effects on fathead minnows. *Environ. Toxicol. Chem.* submitted.

S2. Yi T, Harper W. 2007. The link between nitrification and biotransformation of 17α-ethinylestradiol. *Environ. Sci. Technol.* 41:4311-6.

Environmental Science & Technology

Article

pubs.acs.org/est

Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein

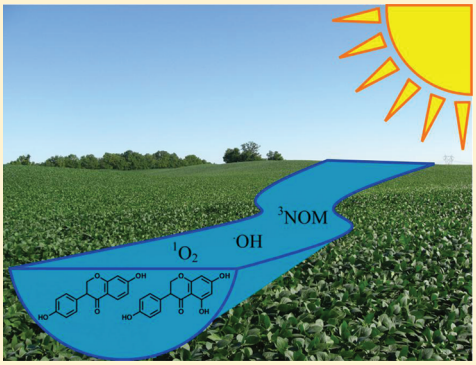
Megan M. Kelly[†] and William A. Arnold*[,†,‡]

[†]Water Resources Science Graduate Program, University of Minnesota, 1985 Buford Avenue, St. Paul, Minnesota 55108

[‡]Department of Civil Engineering, University of Minnesota, 500 Pillsbury Drive Southeast, Minneapolis, Minnesota 55455

Supporting Information

ABSTRACT: Genistein and daidzein are two estrogenic compounds derived from plants, especially legumes. This research begins to explore their environmental fate, focusing on direct and indirect photolysis. UV−visible spectra for both compounds at varying pH values were taken, the pK_a values for both compounds were measured, and UV−visible spectra for each protonation state were determined. The loss of both compounds in deionized water was observed upon exposure to natural sunlight, and the quantum yields were determined for each protonation state. In Mississippi River water, direct photolysis does not account for all of the loss of genistein and daidzein. The mechanism of indirect photolysis was probed using quenchers and sensitizers, and results suggest that daidzein is transformed mainly via direct photolysis and singlet oxygenation, while genistein is transformed mainly via reaction with triplet-state natural organic matter. The parameters determined in this study will allow for estimation of the concentration of genistein and daidzein in sunlit surface waters, which will allow for assessment of any risks posed to aquatic wildlife.



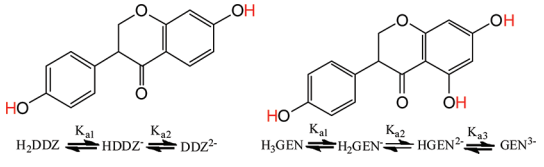
INTRODUCTION

Endocrine disrupting compounds (EDCs) are of serious concern as aquatic pollutants due to the low concentrations at which they are bioactive. Most research on the environmental presence, degradation pathways, and effects on humans and wildlife has focused on natural human estrogens, synthetic estrogens for therapeutic and contraceptive use, or industrially produced EDCs such as bisphenol A and nonylphenol.[1−4] Another class of EDCs, the phytoestrogens (plant-derived estrogens), is also gaining attention. Although typically 2 to 3 orders of magnitude less potent than the human estrogens, such as 17β-estradiol and estrone or the synthetic estrogen 17α-ethinylestradiol, several investigations have indicated that phytoestrogens may act as EDCs.[5−8] Genistein and daidzein (Scheme 1), the focus of this study, are two such compounds.

These phytoestrogens are present at high concentrations in soybean plants and other legumes. Genistein and daidzein were detected in the effluents of soy-processing industries (4.8−151 μg/L and 2.1−98.9 μg/L, respectively), other plant-processing industries (up to 10.5 μg/L and 3.5 μg/L, respectively), and other industries (up to 30.8 μg/L and 12.4 μg/L, respectively).[9,10] Genistein and daidzein have also been detected in a variety of surface waters. In Iowa streams draining large areas of corn and soybean fields and Swiss streams draining pastures and urban lands, genistein was detected at concentrations of 8 ng/L to 24.2 ng/L, and daidzein was detected at concentrations of 6.4 ng/L to 41 ng/L.[11−13] In streams draining urban or residential areas, the concentrations observed have usually been lower (2−7 ng/L daidzein and 4−7 ng/L genistein),[14,15] but in one urban Japanese stream, up to 143.4 μg/L genistein and 42.9 μg/L daidzein were observed.[16] A conclusive explanation of these extremely high concentrations was not offered, but it was noted that there are food and wood pulp factories in the area.

The potential impact of phytoestrogens on aquatic systems merits investigation. This study seeks to determine the importance of photolysis in the environmental fate of genistein and daidzein in natural waters. Other studies have shown that estrogenic compounds with at least one phenolic group, such as nonylphenol, 17β-estradiol, and 17α-ethinylestradiol, undergo direct photolysis slowly but are efficiently transformed by hydroxyl radical (·OH) in engineered UV systems.[17−19] Similarly, bisphenol A has been shown to undergo slow removal by direct photolysis.[20,21] Degradation of bisphenol A is

Scheme 1



Received: January 5, 2012
Revised: April 4, 2012
Accepted: April 11, 2012
Published: April 11, 2012

enhanced by ·OH and triplet state natural organic matter (^{3}NOM).[20,21] Other wastewater-derived compounds with electron-donating groups on an aromatic ring are removed slowly by direct photolysis and more quickly by reaction with ·OH, singlet oxygen ($^1O_2$), or ^{3}NOM.[22−24]

Direct photolysis occurs when a molecule absorbs sunlight and is transformed. The first order rate expression is given by

$$-\frac{dC}{dt} = \phi_{dc} k_a C \quad (1)$$

where C is the concentration of the compound of interest, k_a is the rate at which the compound absorbs light summed over the wavelengths at which it absorbs, and ϕ_{dc} is the quantum yield.[25] The quantum yield represents the fraction of molecules absorbing a photon that are transformed. It is calculated using the equation

$$\phi_{dc} = \frac{k_{dc}}{k_{da}} \sum_{\lambda} \frac{\varepsilon_{\lambda a} L_\lambda \lambda_{range}}{\varepsilon_{\lambda c} L_\lambda \lambda_{range}} \phi_{da} \quad (2)$$

where ϕ_{da} is the quantum yield of a chemical actinometer (a compound with a known quantum yield), k_{dc} and k_{da} are the rate constants for the photolysis of the compound and the actinometer, $\varepsilon_{\lambda a}$ and $\varepsilon_{\lambda c}$ are the molar absorptivities of the actinometer and compound, respectively, at wavelength λ, L_λ is the solar irradiance at wavelength λ, and λ_{Range} is the difference between λ_n and λ_{n+1}.[25]

Sunlight interacting with the organic matter present in natural water also generates transient reactive species such as $^1O_2$, ·OH, ^{3}NOM, or other photochemically produced reactive intermediates. The reaction of these photochemically produced reactive intermediates with pollutants is known as indirect photolysis.

Given their presence in effluent streams and surface waters, photolysis is a potentially important loss process for genistein and daidzein in sunlit aquatic environments. This study sought to quantify the rates of direct and indirect photolysis of genistein and daidzein. Given their structural similarity to other estrogenic compounds and the presence of the phenolic rings, it was hypothesized that genistein and daidzein would be subject to both direct and indirect photolysis processes.

METHODS

Experimental Design. The UV−visible spectrum of each compound was measured at a variety of pH values. This allowed the determination of the pK_a values of each compound and provided information necessary for calculation of direct photolysis quantum yields. Genistein, daidzein, and an actinometer were exposed to natural and simulated sunlight in deionized (DI) water or Mississippi River water (MRW) at a variety of pH values. The actinometer allowed experiments with long exposure times to be conducted without concern for changes in cloud cover or sunlight intensity. Important indirect photolysis processes were identified by quencher and sensitizer experiments.

Chemicals. Chemicals and the MRW used in this study are described in the Supporting Information (SI).

HPLC Methods. High pressure liquid chromatography (HPLC) methods are described in the SI.

UV−Vis Spectroscopy. Stock solutions of genistein (2.5 mM) and daidzein (1 mM) were prepared in methanol. Separately, buffered aqueous solutions from pH 4.5−13.5 were prepared. Stock solutions were diluted with the aqueous buffers resulting in 100 μM daidzein with 10% methanol or 50 μM genistein with 2% methanol. The resulting samples were placed in a 1-cm path length quartz cuvette, and spectra were collected from 190 to 400 nm at each selected pH value by a Shimadzu UV-1601 spectrophotometer. An aqueous blank containing either 10% or 2% methanol was used.

Solar Exposure. To conduct solar irradiation experiments, phytoestrogen and actinometer solutions in quartz tubes (o.d. = 1.3 cm, i.d. = 1.1 cm, V = 10 mL) were set on the roof of the Civil Engineering building at the University of Minnesota in Minneapolis, MN (latitude 45°N), which provided no shadows for most of the day. Exposures of daidzein were conducted at pH 8.75 on Jul 12, 2009 and pH 5.1 and pH 12 on Jul 20, 2009. Experiments with genistein were conducted at pH 8.7 and pH 12 on Jul 20, 2009, pH 11 on Nov 4, 2009, and pH 5 on Nov 6, 2009. Actinometer solutions consisted of 10 μM p-nitroacetophenone (PNAP) with 80.2 mM or 22 mM pyridine or 10.5 μM p-nitroanisole (PNA) with 0.39 mM or 1.41 mM pyridine. Actinometers were selected to have a half-life longer than that of the reaction of interest, using the equations in Leifer.[25] Subsamples were taken throughout the exposure and analyzed by HPLC.

Identifying the Indirect Photolysis Processes. Sensitizers and inhibitors were added to the MRW samples at pH 12 and 8.7 to investigate indirect photolysis processes. Sorbic acid (8.9 mM) was added to quench ^{3}NOM, sodium azide (1.5 mM) was added to quench $^1O_2$, isopropyl alcohol (1% = 131 mM) was added to quench ·OH, or the solution was sparged with nitrogen or argon gas for 5 min to inhibit the formation of $^1O_2$ and/or to potentially allow enhanced formation of ^{3}NOM. Each sample was placed in a quartz tube and exposed to simulated sunlight in an Atlas CPS+ solar simulator with the intensity set to 765 W/m^2. For deoxygenated solutions, the tubes were capped with septa, and the solutions were sampled with a needle and syringe to prevent introduction of oxygen to the system. Subsamples were taken periodically and analyzed by HPLC.

Singlet Oxygen Rate Constant and Sensitization. To quantify the rate constant for the reaction between $^1O_2$ and genistein or daidzein, solutions of 7 μM genistein at pH 8.5, 5.9 μM daidzein at pH 8.75, or 20 μM furfuryl alcohol (FFA; a reference compound with a known rate constant, $k_{1O_2} = 1.2 \times 10^8$ M^{-1} s^{-1}) and 6 μM Rose Bengal, a $^1O_2$ sensitizer, were photolyzed simultaneously.[26,27] Subsamples were taken throughout the experiment and analyzed by HPLC.

To investigate whether genistein and daidzein produce singlet oxygen, solutions of 20 μM FFA were made in either DI water, DI water with 4.1 μM genistein, DI water with 4.3 μM daidzein, MRW, MRW with 6.7 μM genistein, or MRW with 7.1 μM daidzein. The solutions were irradiated in quatz tubes in the solar simulator for 2h, with subsamples removed every 20 min.

Hydroxyl Radical Rate Constant. To quantify the rate constant for the reaction between ·OH and genistein or daidzein, Fenton's reagent (40 μM $FeSO_4 \cdot 7H_2O$ and 1 mM H_2O_2) was added to solutions containing the compound of interest and 20 μM acetophenone ($k_{OH} = 5.9 \times 10^9$ M^{-1} s^{-1}), as a reference compound.[28,29] The solutions were adjusted to pH 3 with sulfuric acid. Samples (0.5 mL) were taken frequently, quenched with 0.5 mL methanol, and analyzed by HPLC.

Data Analysis. Data fitting to determine pK_a values was carried out with Scientist for Windows (v.2.1; Micromath

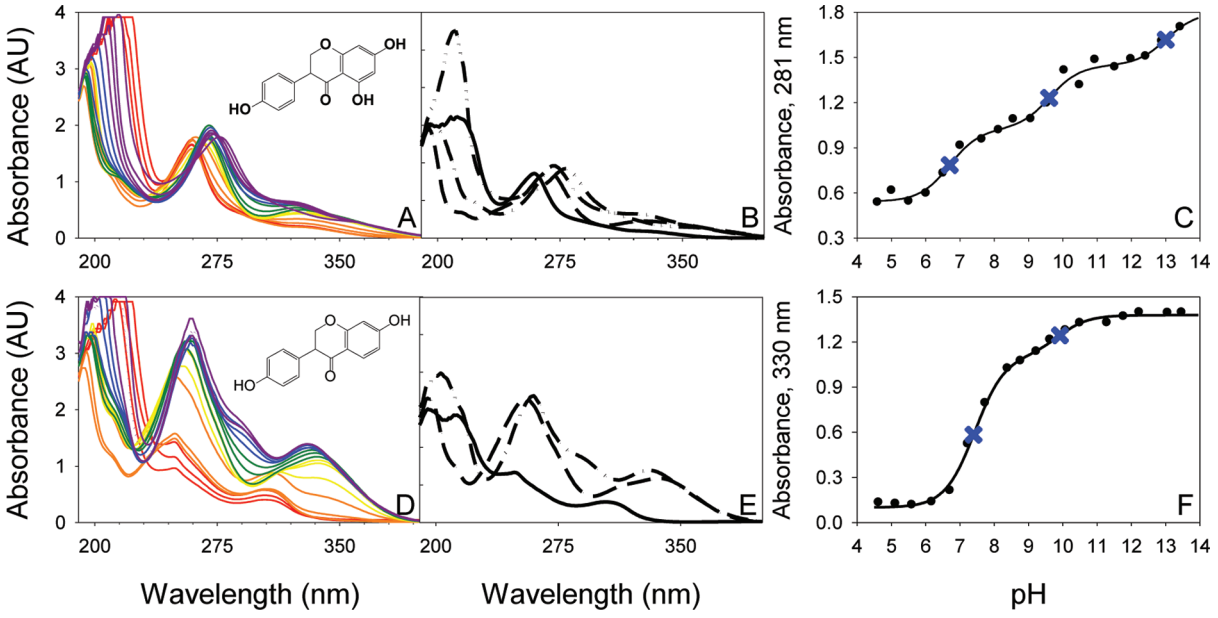


Figure 1. UV−visible spectra collected as a function of pH (panel A for genistein and D for daidzein, pH increases from red to purple) used to determine pK_a values. The pK_a values determined were 6.70 ± 0.39, 9.62 ± 0.45, and 13.0 ± 1.1 for genistein and 7.43 ± 0.10 and 9.88 ± 0.35 for daidzein (indicated by × in C and F), respectively, by observing shifts in the UV−vis spectra. The component spectra (panels B and E) for each protonation state of genistein and daidzein (H_3GEN or H_2DDZ: solid line; H_2GEN^- or $HDDZ^-$: dashed line; $HGEN^{2-}$ or DDZ^{2-}: dash-dot line; GEN^{3-}: dash-dot-dot line) were then determined using a matrix transformation, which is detailed in the Supporting Information.

Scientific Software). Protonation state spectra were determined using MATLAB (v R2009b, Mathworks). Solar spectra were generated using SMARTS (v 2.9.5, http://www.nrel.gov/rredc/smarts/).[30,31] Quantum yields and rate constants were determined via linear regressions using Microsoft Excel 2007.

RESULTS AND DISCUSSION

UV−Vis Spectra. The absorbance spectra obtained as a function of pH are shown in Figure 1. The spectra were used to determine the pK_a values of genistein and daidzein by fitting spectral data to eqs 3−5

$$(\chi_1)(A_{1,\lambda}) + (\chi_2)(A_{2,\lambda}) + (\chi_3)(A_{3,\lambda}) + (\chi_4)(A_{4,\lambda}) = A_\lambda \quad (3)$$

$$A_\lambda = \frac{A_1[H^+]^3 + A_2[H^+]^2K_{a1} + A_3[H^+]K_{a1}K_{a2} + A_4K_{a1}K_{a2}K_{a3}}{[H^+]^3 + [H^+]^2K_{a1} + [H^+]K_{a1}K_{a2} + K_{a1}K_{a2}K_{a3}} \quad (4)$$

$$A_\lambda = \frac{A_1[H^+]^2 + A_2[H^+]K_{a1} + A_3K_{a1}K_{a2}}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \quad (5)$$

where χ_i is the molar fraction of genistein in protonation state i at a given pH, $A_{i,\lambda}$ is the absorbance of that protonation state at the given pH, and A_λ is the total absorbance at wavelength λ at that pH. Eqs 4 and 5 are expanded forms of eq 3, where A_1, A_2, A_3, and A_4 are the absorbances of each of the protonation states, K_{a1}, K_{a2}, and K_{a3}, are the acid−base equilibrium constants, and $[H^+]$ is the concentration of hydrogen ions.[24]

By fitting of eqs 4 (for genistein) and 5 (for daidzein) to the UV−vis spectra of the respective phytoestrogens at various pH values shown in Figure 1, the pK_a values for each compound were determined.[24] The wavelength for the fitting, selected by trial and error, was 330 nm for daidzein and 281 nm for genistein. As shown in Figure 1, the spectrophotometric titration curves at these wavelengths give pK_a values of 6.70 ± 0.39, 9.62 ± 0.45, and 13.0 ± 1.1 for genistein and 7.43 ± 0.10 and 9.88 ± 0.35 for daidzein. Reported errors are 95% confidence intervals.

Determination of Quantum Yields. The quantum yield of photolysis was determined at each pH for each compound using eq 2. The first term in eq 2, $((k_{dc})/(k_{da}))$, was calculated by plotting the concentration of substrate $([C]/[C_0])$ versus the concentration of actinometer $([\text{actinometer}]/[\text{actinometer}]_0)$ on a log−log scale and taking the slope of the resulting plot as $((k_{dc})/(k_{da}))$. These plots, generated using data from the solar exposure experiments, are presented in Figure S1 of the SI. Spectra of genistein and daidzein at each experimental pH were calculated using the component spectra (Figure 1, panels B and E, determined using the method of Boreen et al.[24,32] as described in the SI). This was done because the samples for photolysis and the samples for the UV−vis spectra were not adjusted to exactly the same pH values. Spectra and quantum yields of the actinometers were those reported by Leifer.[25] Solar irradiance spectra were generated for each experimental date using SMARTS,[30,31] and the overlap integral of the solar spectra with genistein, daidzein, or the actinometer spectrum was calculated.

The quantum yields determined at each experimental pH are found in Table 1. Genistein reacted more quickly at high pH and exhibited the largest quantum yield at pH 12 (2.9×10^{-4}). The spectral overlap integral for genistein, however, also increases with increasing pH, so the next highest quantum yield was at pH 5 (1.3×10^{-4}). The lowest quantum yield for genistein was observed at pH 8.7 (9.4×10^{-6}). Daidzein reacted most quickly at pH 8.7 and also exhibited the largest quantum yield at pH 8.7 (6.6×10^{-4}). At pH 12, daidzein exhibited its smallest quantum yield (1.4×10^{-4}). Reaction rate depends on both light absorbance rate and quantum yield, and light absorbance depends strongly on pH. Thus, at a given pH, it is possible for a protonation state present as a minor fraction

dx.doi.org/10.1021/es300041f | Environ. Sci. Technol. 2012, 46, 5396−5403

Table 1. Quantum Yields for Direct Photolysis at Tested pH Values, Second-Order Rate Constants for Reactions with Hydroxyl Radical and Singlet Oxygen, and Calculated Quantum Yields for Individual Protonation States

Observed				
	pH	ϕ_{dc} (-)	k_{OH} (M^{-1} s^{-1})	$k_{^1O_2}$ (M^{-1} s^{-1})
genistein	5	1.3×10^{-4}	$8.73 \pm 0.31^{a,b} \times 10^9$	$3.57 \pm 0.08 \times 10^{7c}$
	8.7	9.4×10^{-6}		
	11	7.2×10^{-5}		
	12	2.9×10^{-4}		
daidzein	5	4.7×10^{-4}	$6.94 \pm 0.09^{b} \times 10^9$	$1.84 \pm 0.03 \times 10^{7c}$
	8.7	6.6×10^{-4}		
	12	1.4×10^{-4}		

Calculated			
protonation state	ϕ_{dc} (-)	protonation state	ϕ_{dc} (-)
H_3GEN	6.9×10^{-5}	H_2DDZ	3.9×10^{-4}
H_2GEN^-	3.3×10^{-6}	$HDDZ^-$	7.5×10^{-4}
$HGEN^{2-}$	5.0×10^{-5}	DDZ^{2-}	3.1×10^{-5}
GEN^{3-}	3.2×10^{-3}		

[a]Reported errors are 95% confidence intervals. [b]Reactions with Fenton's reagent to determine the hydroxyl radical rate constant were conducted at pH 3. [c]Photolyses with Rose Bengal to determine the singlet oxygen rate constant were conducted at pH 8.5.

to strongly influence the total quantum yield of genistein or daidzein if the product of light absorbance rate and quantum yield is dramatically greater than that of the major protonation state present.

Determination of Component Quantum Yields. Because genistein and daidzein both consist of more than one protonation state at environmentally relevant pH values, it is useful to consider that the total rate constant of transformation is equal to the sum of the rate constants for each protonation state

$$k_{dc,total} = k_{dc,\chi_1} + k_{dc,\chi_2} + k_{dc,\chi_3} + k_{dc,\chi_4} \quad (6)$$

These values can all be divided by k_{da}, which yields a relative rate value $(k_{dc,\chi_i})/(k_{da})$ that can be used to calculate the quantum yield of each protonation state. Eq 7 was used to determine $(k_{dc,\chi_i})/(k_{da})$

$$\begin{bmatrix} \chi_{1,\,pH\,5} & \cdots & \chi_{4,pH\,5} \\ \vdots & \ddots & \vdots \\ \chi_{1,pH\,12} & \cdots & \chi_{4,pH\,12} \end{bmatrix} \begin{bmatrix} \frac{k_{dc,\,\chi_1}}{k_{da}} \\ \vdots \\ \frac{k_{dc,\,\chi_4}}{k_{da}} \end{bmatrix} = \begin{bmatrix} \frac{k_{dc,\,total,\,pH\,5}}{k_{da}} \\ \vdots \\ \frac{k_{dc,\,total,\,pH\,12}}{k_{da}} \end{bmatrix} \quad (7)$$

where $(k_{dc,\chi_i})/k_{da}$ is the rate of transformation of protonation state i relative to the transformation of the actinometer used to measure $(k_{dc,total})/k_{da}$, the observed rate of transformation of genistein or daidzein at the indicated pH normalized to the actinometer.[24] For this analysis, solar exposures needed to be conducted using the same actinometer for all pH values to compare $(k_{dc,total})/k_{da}$ values. At pH 5, however, genistein did not react quickly enough to be compared to the same actinometer as the other pH values. To estimate the $((k_{dc})/(k_{da}))$ value for genistein at pH 5, k_{da} was calculated for the PNAP actinometer with 22 mM pyridine, and for the PNA actinometer with 0.39 mM pyridine according to the equation

$$k_{da} = \phi_{da} \sum_{\lambda} \varepsilon_\lambda L_\lambda \quad (8)$$

where $\Sigma_\lambda \varepsilon_\lambda L_\lambda$ is the overlap of the absorbance spectrum of the chemical and the solar spectrum.[25] The ratio of the two k_{da} values can be used to convert the $((k_{dc})/(k_{da}))$ for genistein at pH 5 from a PNAP actinometer basis to a PNA actinometer basis. The $((k_{dc})/(k_{da}))$ value for genistein at pH 5 normalized to the PNA actinometer is then compared to those for genistein at the other pH values tested. The solar spectrum from Nov 6, 2009 was used for the PNAP actinometer, and the solar spectrum from Jul 20, 2009 was used for the PNA actinometer. The $k_{da,PNAP}/k_{da,PNA}$ conversion factor was calculated to be 0.0405. The quantum yields for each component species are found in Table 1. Using the component spectra and the component quantum yields, the fraction of direct photolysis attributable to each protonation state can be determined, according to eq 1.

As shown in Table 1, the fully deprotonated species of genistein has the highest quantum yield, but under typical environmental pH conditions, it is not present at high enough concentrations to contribute substantially to the rate of direct photolysis (Figure 2A). The fully protonated species of genistein dominates the contribution to the rate of direct photolysis at pH values of 7 and below. The singly deprotonated species of genistein dominates the direct photolysis at pH values near its maximum molar fraction. The singly protonated species of genistein dominated at pH values from 9 to 11.

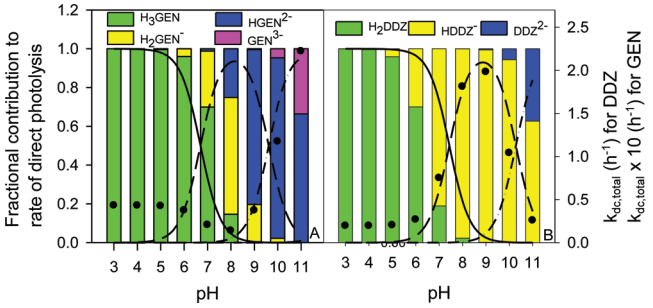


Figure 2. Contributions by each protonation state of genistein (A) and daidzein (B) to the overall direct photolysis rate (colored bars) and fractional concentrations of each as a function of pH (H_3GEN or H_2DDZ: solid line; H_2GEN^- or $HDDZ^-$: dashed line; $HGEN^{2-}$ or DDZ^{2-}: dash-dot line; GEN^{3-}: not visible, fractional concentration < 0.01 at pH 11). The circles are the overall rate constant $(k_{dc,total})$ calculated based on the quantum yields and absorbance spectra of each protonation state as a function of pH using eq 6 and the solar spectrum from ref 25 for summer 40°N.

The singly deprotonated species of daidzein has the highest quantum yield and dominates direct photolysis rates at pH 7 and above (Figure 2B). The fully protonated species of daidzein dominates direct photolysis below pH 7. The contribution of each species is mainly dependent on the pH of the solution but is also influenced to a lesser extent by the quantum yield of each species. Also shown in Figure 2 are the calculated rate constants of direct photolysis $(k_{dc,total})$ at each pH value based on the quantum yields and absorbance spectra for each protonation state substituted into eq 6 and using the solar spectrum for summer 40°N from ref 25.

Hydroxyl Radical Rate Constant. Concentrations of genistein, daidzein, and acetophenone were monitored during

dx.doi.org/10.1021/es300041f | Environ. Sci. Technol. 2012, 46, 5396−5403

their reaction with Fenton's reagent. These experiments were carried out at pH 3 to prevent iron precipitation. The rate constant was determined by plotting the fractional concentration of genistein or daidzein against the fractional concentration of the acetophenone on a log−log scale (Figure S2) and taking the slope to be $(\ln([C]/[C_0]))/(\ln([R]/[R_0]))$ in the equation

$$k_{ROS,C} = \frac{\ln\left(\frac{[C]}{[C_0]}\right)}{\ln\left(\frac{[R]}{[R_0]}\right)} k_{ROS,R} \quad (9)$$

where $k_{ROS,R}$ is the rate constant for the reaction between acetophenone and the hydroxyl radical, and $k_{ROS,C}$ is the rate constant for the reaction between genistein or daidzein and the hydroxyl radical. The rate constants for the reactions of hydroxyl radical and genistein or daidzein were determined to be $8.71 \pm 0.31 \times 10^9$ M^{-1} s^{-1} and $6.94 \pm 0.09 \times 10^9$ M^{-1} s^{-1}, respectively (Table 1; Figure S2, SI). These values are comparable to those observed for other phenolic EDCs (bisphenol A, 17α-ethinylestradiol, and 17β-estradiol; 3.3×10^9 M^{-1} s^{-1}−1.14×10^{10} M^{-1} s^{-1}, pH 6.8).[18,33,34]

Singlet Oxygen Rate Constant. To determine the singlet oxygenation rate constants, the concentrations of genistein, daidzein, and FFA were monitored during irradiation in the presence of Rose Bengal at pH 8.5. Instead of acetophenone, the concentration of FFA was used to determine the value of $(\ln([C]/[C_0]))/(\ln([R]/[R_0]))$. The measured rate constants for the reactions of singlet oxygen with genistein or daidzein were $3.57 \pm 0.08 \times 10^7$ M^{-1} s^{-1} and $1.84 \pm 0.03 \times 10^7$ M^{-1} s^{-1}, respectively (Table 1; Figure S3, SI). These values are nearly an order of magnitude lower than the rate constant reported for bisphenol A in pH 10 water, 1.01×10^8 M^{-1} s^{-1}.[35] Bisphenol A at pH 10 would consist of 27% of the fully protonated form, 70% of the singly deprotonated form, and 3% of the fully deprotonated form. These experiments were carried out at pH ∼8.5, where the singly deprotonated species is the dominant protonation state for both genistein and daidzein. That the rate constant for bisphenol A is nearly an order of magnitude greater suggests that the singly deprotonated form of bisphenol A is more reactive toward singlet oxygen than that of either genistein or daidzein. As pH increases, the singlet oxygen rate constant may also increase, because the aromatic rings become more electron-rich when additional deprotonation steps occur.

Importance of Indirect Photolysis Processes. Side-by-side irradiation of solutions of genistein or daidzein in DI water and MRW showed that direct photolysis could not account for all observed losses in MRW at all pH values tested, as shown in Figure 3 for pH 8.5 and in Figure S1 for other pH values. To determine the mechanism of indirect photolysis, genistein and daidzein were exposed to simulated sunlight in the presence of probes and quenchers (Figure 3). When added to a pH 8.5 solution of MRW and genistein, isopropanol slightly slowed the loss of genistein ($k_{isopropanol}/k_{MRW} = 0.85$). Deoxygenation also slightly slowed the reaction ($k_{deoxygenated}/k_{MRW} = 0.86$), but adding sodium azide caused genistein transformation to occur more rapidly ($k_{sodium\ azide}/k_{MRW} = 1.46$), an unexpected effect seen previously for mefenamic acid.[22] The reaction of genistein in MRW in the presence of sorbic acid ($k_{sorbic\ acid}/k_{MRW} = 0.04$) slowed dramatically, and the resulting rate of loss was slower than that in DI water ($k_{DI}/k_{MRW} = 0.11$). The reaction of daidzein in MRW was unaffected by sodium azide ($k_{sodium\ azide}/k_{MRW} = 0.92$) and isopropanol ($k_{isopropanol}/k_{MRW} = 1$).

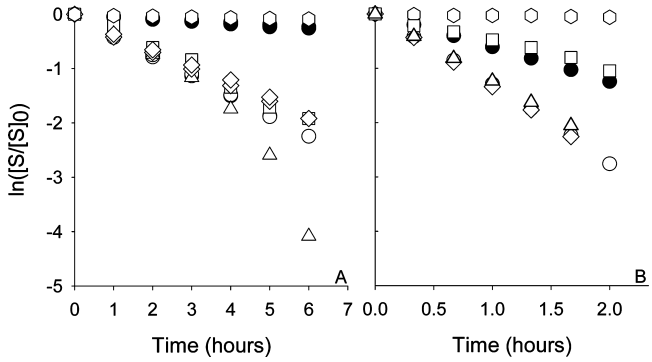


Figure 3. Loss of genistein (A) and daidzein (B) in DI water (filled circles), MRW (open circles), deoxygenated MRW (squares), MRW with sodium azide (triangles), MRW with isopropanol (diamonds), and MRW with sorbic acid (hexagons).

Deoxygenation of the MRW solution slowed the reaction of daidzein substantially ($k_{deoxygenated}/k_{MRW} = 0.37$), so it was slightly slower than a solution of daidzein in DI water ($k_{DI}/k_{MRW} = 0.46$). Sorbic acid slowed the loss of daidzein in MRW to a rate even slower ($k_{sorbic\ acid}/k_{MRW} = 0.02$) than the rate in DI water.

Sorbic acid is known to quench ^{3}NOM, so these results suggest that ^{3}NOM may play a role in the indirect photolysis of genistein and daidzein. To a lesser extent, sorbic acid may also be a quencher of $^1O_2$ ($k < 5 \times 10^7$ M^{-1} s^{-1}), but given the minor effects of the other quenchers on genistein, it seems most likely that $^1O_2$ and ·OH are not important and that ^{3}NOM is largely responsible for the observed reaction enhancement in MRW.[36,37] In the case of daidzein, it seems that $^1O_2$ may play a role, because deoxygenating the MRW slows the reaction to the rate at which it proceeds in DI water. It is unclear why azide did not quench the singlet oxygenation, but oxygen may play an additional role (see below). Adding sorbic acid, however, to either genistein or daidzein in MRW slows the reaction down to an even slower rate than that which occurs in DI water.

To further explore this issue, genistein and daidzein were exposed to sunlight in deoxygenated DI water, or in DI water in the presence of sorbic acid, both at pH 8.5. The screening of sunlight by sorbic acid accounts for a 6% decrease in rate constant (data not shown). The results in Figure 4 demonstrate that sorbic acid quenches the direct photolysis of genistein and daidzein in DI water. Thus, this explains why the sorbic acid slows the reaction in MRW to a rate slower than that in DI water: the sorbic acid is quenching direct photolysis as well as any reaction with ^{3}NOM. This indicates that the direct photolysis of these two phytoestrogens proceeds through a triplet excited state. The deoxygenated experiments also showed a slower rate of transformation for both genistein and daidzein, indicating either possible self-sensitization of singlet oxygen generation by genistein or daidzein or that oxygen is somehow otherwise involved in the direct photolysis reaction.

To investigate whether genistein and daidzein sensitize the formation of singlet oxygen, FFA was exposed to simulated sunlight in the presence of DI water with and without genistein or daidzein and MRW with and without genistein or daidzein. FFA in the solutions containing genistein or daidzein was removed at the same rate as in the solutions without genistein or daidzein (Figure 5). These results indicate that these

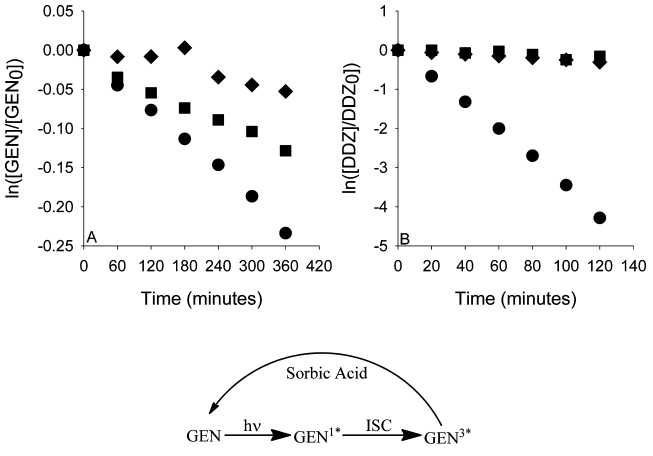


Figure 4. Photolysis of genistein (A) and daidzein (B) in DI water with (diamonds) and without (circles) sorbic acid present and in deoxygenated DI water (squares). The schematic shows the quenching effect of sorbic acid on triplet genistein (which would be equivalent for daidzein). While the quenching effect of sorbic acid suggests a role for the triplet excited state in direct photolysis, the slowing of the rate upon deoxygenation also indicates a role for oxygen in the direct photolysis process.

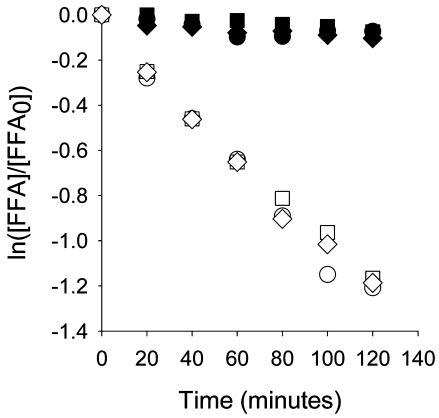


Figure 5. Degradation of furfuryl alcohol in the absence (circles) or presence of genistein (diamonds) or daidzein (squares), in DI water (solid), or MRW (outlined).

phytoestrogens do not serve as singlet oxygen sensitizers. Either the triplet states do not have sufficient energy to generate singlet oxygen or they are too short-lived to produce singlet oxygen (i.e., once formed via intersystem crossing, a chemical bond is broken, completing the direct photolysis step). The latter explanation is consistent with the dramatic quenching effect of sorbic acid on the direct photolysis rate. The slowing of the reaction in DI water in the absence of oxygen (meaning oxygen is not acting as a triplet quencher) and the lack of production of singlet oxygen by irradiation of genistein and daidzein suggests that either additional reactive oxygen species are produced during the photolysis of these phytoestrogens or that oxygen is involved in the direct photolysis reaction of these compounds.

Predicted Photolysis Half-lives. The half-life of direct photolysis of daidzein in sunlit summer and winter near-surface waters was estimated using eq 10[25]

$$t_{1/2} = \ln(2)\left[\sum_{\lambda} \varepsilon_{\lambda,H_2DDZ} \times L(\lambda) \times \Phi_{H_2DDZ} + \sum_{\lambda} \varepsilon_{\lambda,HDDZ^-} \times L(\lambda) \times \Phi_{HDDZ^-} + \sum_{\lambda} \varepsilon_{\lambda,DDZ^{2-}} \times L(\lambda) \times \Phi_{DDZ^{2-}}\right] \quad (10)$$

To estimate the half-life of genistein, the same equation was used, with an additional term in the denominator to account for the additional protonation state in genistein. For both equations, L was assumed to be at 40 ° N latitude, taken from Leifer (sunlight intensity averaged over 24 h).[25] For daidzein at pH 8.5, the half-life was 0.014 days (0.34 h) in summer and 0.046 days (1.1 h) in winter. For genistein at pH 8.5, the half-life was 1.48 days (35.5 h) in the summer and 4.91 days (117.8 h) in the winter. When samples were irradiated in natural sunlight in MRW at pH 8.7, a half-life of 10 h was observed for genistein and a half-life of 1.1 h was observed for daidzein (data not shown).

Assuming a steady state concentration of $^1O_2$ ($[^1O_2]_{ss}$) of 10^{-13} M, an environmentally relevant value,[38] and multiplying by the second order rate constants for genistein or daidzein determined at pH 8.5 gives pseudo-first order rate constants of 3.57×10^{-6} s^{-1} (half-life of 53.9 h) and 1.84×10^{-6} s^{-1} (half-life of 104.6 h), respectively. These half-lives cannot be directly compared to the half-lives of direct photolysis because they do not account for the variable sunlight intensity throughout a day. Loss of genistein is more likely to occur via direct photolysis than reaction with singlet oxygen under conditions with similar steady-state concentrations of singlet oxygen. Loss of daidzein occurs much more quickly via direct photolysis than reaction with singlet oxygen, but singlet oxygen may play a role (Figure 3B).

For hydroxyl radical typical values are on the order of 10^{-17} to 10^{-15} M.[38] The pseudofirst order rate constants would then be on the order of 10^{-8} s^{-1} to 10^{-6} s^{-1} (half-lives of 190–19,000 h). Under circumstances with high concentrations of hydroxyl radical, such as high-nitrate waters, reaction with hydroxyl radical could become an important loss process for genistein and daidzein. The contribution of ^{3}NOM to loss of genistein and daidzein is harder to address because NOM can act as an oxidant or as an antioxidant.[39] If ^{3}NOM were the most important loss process, we would expect to see a decreased rate of transformation in the presence of sorbic acid and an increased rate of transformation under deoxygenated conditions. The effects of sorbic acid or deoxygenation on daidzein, shown in Figures 3 and 4, suggest that while ^{3}NOM may have a limited role for daidzein, reaction with an oxygen-based species is the dominant indirect photolysis process. The effects of sorbic acid are complicated, though, by its quenching of both ^{3}NOM and direct photolysis. The picture is clearer for genistein. Genistein in pH 8.7 MRW exposed to natural sunlight had a half-life of about 10 h (data not shown), substantially faster than the predicted half-life of direct photolysis or reaction with singlet oxygen or hydroxyl radical. This reaffirms that reaction with ^{3}NOM is an important loss process for genistein in surface waters. It is possible that reactive oxygen species other than singlet oxygen are involved, given that deoxygenating samples of genistein or daidzein decreases the rate of transformation, sodium azide does not

dx.doi.org/10.1021/es300041f | Environ. Sci. Technol. 2012, 46, 5396–5403

decrease the rates, and the transformation of FFA is not sensitized by genistein or daidzein. Another possibility is that reaction with oxygen is a necessary step in the direct photolysis process. The role of oxygen in the photolysis of these compounds needs to be investigated further.

The parameters developed in this study will help in estimating the concentration of genistein and daidzein in sunlit surface waters subject to inputs of genistein and daidzein. Estimating the concentrations will help assess the risk to aquatic life. Genistein may be lost at rates on the order of days to weeks, while daidzein may be lost at rates on the order of hours to days. Further research is needed to determine whether other loss processes, such as sorption to solids or biodegradation, are important for genistein and daidzein and to determine if the transformation products retain estrogenicity.

■ ASSOCIATED CONTENT

Supporting Information

Additional text and three figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 612-625-8582. Fax: 612-626-7750. E-mail: arnol032@umn.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We wish to thank Abbey Kopan, Kayla Zielinski, Chris Ryan, Teng Zeng, and Greg LeFevre for their assistance with laboratory work. We also thank the three reviewers for their helpful comments. Funding for this project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR) and the University of Minnesota Graduate School. Ancillary support came from the U.S. Environmental Protection Agency Great Lakes National Priority Office.

■ REFERENCES

(1) Kolpin, D.; Furlong, E.; Meyer, M. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202–1211.

(2) Colborn, T.; vom Saal, F.; Soto, A. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **1993**, *101*, 378–384.

(3) Khanal, S. K.; Xie, B.; Thompson, M. L.; Sung, S.; Ong, S. K.; Van Leeuwen, J. H. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ. Sci. Technol.* **2006**, *40*, 6537–6546.

(4) Sharma, V. K.; Anquandah, G. A. K.; Yngard, R. A.; Kim, H.; Fekete, J.; Bouzek, K.; Ray, A. K.; Golovko, D. Nonylphenol, octylphenol, and bisphenol-A in the aquatic environment: a review on occurrence, fate, and treatment. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* **2009**, *44*, 423–42.

(5) Ardia, D. R.; Clotfelter, E. D. The novel application of an immunological technique reveals the immunosuppressive effect of phytoestrogens in Betta splendens. *J. Fish. Biol.* **2006**, *68*, 144–149.

(6) Clotfelter, E. D.; Rodriguez, A. C. Behavioral changes in fish exposed to phytoestrogens. *Environ. Pollut.* **2006**, *144*, 833–839.

(7) Kiparissis, Y.; Balch, G. C.; Metcalfe, T. L.; Metcalfe, C. D. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka *Oryzias latipes*. *Environ. Health Perspect.* **2003**, *111*, 1158–1163.

(8) Choi, S. Y.; Ha, T. Y.; Ahn, J. Y.; Kim, S. R.; Kang, K. S.; Hwang, I. K.; Kim, S. Estrogenic activities of isoflavones and flavones and their structure-activity relationships. *Planta Med.* **2008**, *74*, 25–32.

(9) Lundgren, M.; Novak, P. J. Quantification of phytoestrogens in industrial waste streams. *Environ. Toxicol. Chem.* **2009**, *28*, 2318–2323.

(10) Kiparissis, Y.; Hughes, R.; Metcalfe, C. Identification of the isoflavonoid genistein in bleached kraft mill effluent. *Environ. Sci. Technol.* **2001**, *35*, 2423–2427.

(11) Kolpin, D.; Hoerger, C.; Meyer, M. Phytoestrogens and mycotoxins in Iowa streams: an examination of underinvestigated compounds in agricultural basins. *J. Environ. Qual.* **2010**, *39*, 2089–2099.

(12) Erbs, M.; Hoerger, C. C.; Hartmann, N.; Bucheli, T. D. Quantification of six phytoestrogens at the nanogram per liter level in aqueous environmental samples using $^{13}C_3$-labeled internal standards. *J. Agric. Food Chem.* **2007**, *55*, 8339–8345.

(13) Hoerger, C. C.; Wettstein, F. E.; Hungerbühler, K.; Bucheli, T. D. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ. Sci. Technol.* **2009**, *43*, 6151–6157.

(14) Kang, J.; Price, W. E.; Hick, L. A. Simultaneous determination of isoflavones and lignans at trace levels in natural waters and wastewater samples using liquid chromatography/electrospray ionization ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2411–2418.

(15) Laganà, A.; Bacaloni, A.; De Leva, I.; Faberi, A.; Fago, G.; Marino, A. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Anal. Chim. Acta* **2004**, *501*, 79–88.

(16) Kawanishi, M.; Takamura-Enya, T.; Ermawati, R.; Shimohara, C.; Sakamoto, M.; Matsukawa, K.; Matsuda, T.; Murahashi, T.; Matsui, S.; Wakabayashi, K.; Watanabe, T.; Tashiro, H. Y.; Yagi, T. Detection of genistein as an estrogenic contaminant of river water in Osaka. *Environ. Sci. Technol.* **2004**, *38*, 6424–6429.

(17) Canonica, S.; Meunier, L.; von Gunten, U. Phototransformation of selected pharmaceuticals during UV treatment of drinking water. *Water Res.* **2008**, *42*, 121–128.

(18) Rosenfeldt, E. J.; Linden, K. G. Degradation of endocrine disrupting chemicals bisphenol A, ethinyl estradiol, and estradiol during UV photolysis and advanced oxidation processes. *Environ. Sci. Technol.* **2004**, *38*, 5476–5483.

(19) Neamtu, M.; Frimmel, F. Photodegradation of endocrine disrupting chemical nonylphenol by simulated solar UV-irradiation. *Sci. Total Environ.* **2006**, *369*, 295–306.

(20) Chin, Y.-P.; Miller, P. L.; Zeng, L.; Cawley, K.; Weavers, L. K. Photosensitized degradation of bisphenol A by dissolved organic matter. *Environ. Sci. Technol.* **2004**, *38*, 5888–5894.

(21) Zhan, M.; Yang, X.; Xian, Q.; Kong, L. Photosensitized degradation of bisphenol A involving reactive oxygen species in the presence of humic substances. *Chemosphere* **2006**, *63*, 378–386.

(22) Werner, J.; McNeill, K.; Arnold, W. Environmental photodegradation of mefenamic acid. *Chemosphere* **2005**, *58*, 1339–1346.

(23) Lam, M.; Young, C. Aqueous photochemical reaction kinetics and transformations of fluoxetine. *Environ. Sci. Technol.* **2005**, *39*, 513–522.

(24) Boreen, A. L.; Arnold, W. A.; McNeill, K. Photochemical fate of sulfa drugs in the aquatic environment: Sulfa drugs containing five-membered heterocyclic groups. *Environ. Sci. Technol.* **2004**, *38*, 3933–3940.

(25) Leifer, A. *The Kinetics of Environmental Aquatic Photochemistry: Theory and Practice*; American Chemical Society: 1988; Vol. *54*, p. 304.

(26) Latch, D. E.; Stender, B. L.; Packer, J. L.; Arnold, W. A.; McNeill, K. Photochemical fate of pharmaceuticals in the environment: cimetidine and ranitidine. *Environ. Sci. Technol.* **2003**, *37*, 3342–3350.

(27) Scully, F. E., Jr.; Hoigné, J. Rate constants for reactions of singlet oxygen with phenols and other compounds in water. *Chemosphere* **1987**, *16*, 681–694.

(28) Buxton, G.; Greenstock, C.; Helman, W. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513−886.
(29) Tang, W.; Huang, C. 2, 4-dichlorophenol oxidation kinetics by Fenton's reagent. *Environ. Technol.* **1996**, *17*, 1371−1378.
(30) Gueymard, C. Parameterized transmittance model for direct beam and circumsolar spectral irradiance. *Sol. Energy* **2001**, *71*, 325−346.
(31) Gueymard, C. A. *SMARTS, a simple model of the atmospheric radiative transfer of sunshine: Algorithms and performance assessment*; Technical Report No. FSEC-PF-270-95; Florida Solar Energy Center: Cocoa, FL, 1995. http://www.fsec.ucf.edu/en/publications/pdf/FSEC-PF-270-95.pdf (accessed December 22, 2011).
(32) Boreen, A. L.; Arnold, W. A.; McNeill, K. Triplet-sensitized photodegradation of sulfa drugs containing six-membered heterocyclic groups: Identification of an SO_2 extrusion photoproduct. *Environ. Sci. Technol.* **2005**, *39*, 3630−3638.
(33) Felis, E.; Ledakowicz, S.; Miller, J. Degradation of Bisphenol A using UV and UV/H_2O_2 processes. *Water Environ. Res.* **2011**, *83*, 2154−2158.
(34) Baeza, C.; Knappe, D. Transformation kinetics of biochemically active compounds in low-pressure UV Photolysis and UV/H_2O_2 advanced oxidation processes. *Water Res.* **2011**, *45*, 4531−4543.
(35) Barbieri, Y.; Massad, W. A.; Díaz, D. J.; Sanz, J.; Amat-Guerri, F.; García, N. A. Photodegradation of bisphenol A and related compounds under natural-like conditions in the presence of riboflavin: kinetics, mechanism and photoproducts. *Chemosphere* **2008**, *73*, 564−571.
(36) Velosa, A. C.; Baader, W. J.; Stevani, C. V.; Mano, C. M.; Bechara, E. J. H. 1,3-Diene probes for detection of triplet carbonyls in biological systems. *Chem. Res. Toxicol.* **2007**, *20*, 1162−1169.
(37) Buffle, M. O.; von Gunten, U. Phenols and amine induced HO generation during the initial phase of natural water ozonation. *Environ. Sci. Technol.* **2006**, *40*, 3057−3063.
(38) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*, 2nd ed.; John Wiley & Sons: Hoboken, NJ, 2003; Chapter 16.
(39) Wenk, J.; von Gunten, U.; Canonica, S. Effect of dissolved organic matter on the transformation of contaminants induced by excited triplet states and the hydroxyl radical. *Environ. Sci. Technol.* **2011**, *45*, 1334−1340.

Running Head: phytoestrogen environmental presence and biological effects

***Corresponding author:** Heiko L. Schoenfuss

St. Cloud State University

Aquatic Toxicology Laboratory, WSB-273

720 Fourth Avenue South

St. Cloud, MN 56301, USA

(USA) 320-308-3130 (phone)

(USA) 320-308-4166 (fax)

hschoenfuss@stcloudstate.edu

Total number of words in text, references, tables and figure legends: **5646**

PHYTOESTROGENS IN THE ENVIRONMENT: I. OCCURRENCE AND EXPOSURE EFFECTS ON FATHEAD MINNOWS

DANIEL C. REARICK †, NATHAN FLEISCHHACKER ‡, MEGAN M. KELLY §, WILLIAM A. ARNOLD ‡§ , PAIGE J. NOVAK ‡ §, AND HEIKO L. SCHOENFUSS †*

† Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

Abstract

Naturally occurring phytoestrogens may mimic biogenic estrogens and modulate endocrine action in vertebrates. Little is known about their temporal and spatial variability in the environment and the biological effects associated with exposures. This study assessed the environmental presence of phytoestrogens in human-impacted and relatively pristine areas. The response in larval and sexually mature fathead minnows to environmentally relevant concentrations of three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture was also quantified. Phytoestrogens were only present in the human-impacted surface waters. When detected, mean concentrations were low, (±standard deviation) 1.4 ± 0.5 ng/L, 1.6 ± 0.7 ng/L, and 1.1 ± 0.2 ng/L for genistein, daidzein, and formononetin, respectively, in an urban lake, and 1.6 ± 0.4 ng/L, 1.8 ± 1.3 ng/L, and 2.0 ng/L in treated wastewater effluent. Biochanin A was detected twice while zearalenone and coumestrol were never detected. No clear temporal trends of aqueous phytoestrogen concentration were evident. Larval survival was significantly reduced in genistein, formononetin and mixture treatments while adult male fish only exhibited subtle changes to their anatomy, physiology and behavior. Daidzein exposed adult females produced greater quantities of eggs. This research indicates that genistein, daidzein, and formononetin are likely rapidly attenuated and are unlikely to cause widespread ecological harm in the absence of other stressors.

Keywords: endocrine-active compounds; reproduction; behavior; *Pimephales promelas*

INTRODUCTION

Numerous studies have evaluated sublethal consequences for androgenic, estrogenic and anti-androgenic contaminants originating from agricultural runoff, municipal wastewater and industrial effluent [reviewed by 1,2]. These effects range from reproductive impairments and skewed sex ratios in adult fish [3-6] to reduced locomotion and predator avoidance performance in larval fish [7-9]. Less attention, however, has been focused on naturally occurring phytoestrogens with similar endocrine modulating capabilities. Analogous to biogenic estrogen counterparts, phytoestrogens can influence development [10-14], alter behavior [11,15,16], and impair reproductive success [10-17] in individuals, with population significance yet to be established. Further understanding of biological responses following phytoestrogen exposure singly and in mixtures across adult and larval life stages will aid in assessing deleterious contributions of phytoestrogens to aquatic life.

Despite possible effects on wild populations of fish, the presence of phytoestrogens in the environment, both in human-impacted and relatively pristine surface waters, is still widely unknown. Several studies have assessed phytoestrogen occurrence downstream from pulp and paper mill discharges, finding elevated concentrations of isoflavones and phytosterols [4,18-20]. Municipal and industrial effluents are also highly variable sources of phytoestrogens to surface water, containing concentrations ranging from 1 to 250,000 ng/L genistein plus daidzein [14,21-23]. Non-point agricultural runoff can also contribute phytoestrogens to surface water, although such runoff generally contains much lower concentrations of phytoestrogens (1-10 ng/L) [24,25]. Combined or individually, these sources could convey significant quantities of

phytoestrogens to aquatic ecosystems. It is not known, however, whether and at what concentrations phytoestrogens are present in most surface waters.

Given the remaining uncertainty regarding the environmental presence and biological effects of phytoestrogen exposure on fish species such as the fathead minnow (*Pimephales promelas*), two major objectives were evaluated during this study. First, environmental presence of phytoestrogens in human-impacted and relatively pristine areas was assessed. Second, organismal response (larval and sexually mature fish) was quantified following exposure to three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture at concentrations relevant to environmental exposures. Taken together, these objectives allow for much clearer assessment of phytoestrogen risk to ecologically relevant fish species based on their environmental presence, apparent persistence, and biological effects.

METHODS

Surface water samples

Sample collection. To understand the environmental concentrations of phytoestrogens, surface water samples were taken at five sites in the Upper Midwest. Three sites: Lake Vadnais (Vadnais Heights, MN), the Metropolitan Wastewater Treatment Plant (Metro Plant) effluent channel (St. Paul, MN) and Straight Lake (Straight Lake State Park, WI) focused on the temporal variability of phytoestrogens over two sampling campaigns (February 5, 2011 to July 21, 2011 and May 5, 2012 to June 1, 2012 for Lake Vadnais, May 10, 2011 to September 19, 2011 and May 5, 2012 to June 1, 2012 for the Metro Plant, and April 25, 2011 to October 30, 2011 and May 5,

2012 to May 13, 2012 for Straight Lake), during which samples were taken in triplicate. The remaining two sites, Minnesota River (Mankato, MN) and Okabena Creek (Brewster, MN) were sampled intensely on three (two for the Minnesota River and one for Okabena Creek) occasions for spatial variation in phytoestrogen concentrations upstream and downstream from suspected phytoestrogen point sources. Samples from the upstream locations, the suspected phytoestrogen discharges themselves, and 1-2 points downstream of the discharges were sampled in triplicate.

Samples were collected from the top 0.5-meter of the water column in solvent-rinsed 1-L amber glass containers. Upon collection, all samples were packed on ice for transport to the laboratory where they were refrigerated at 4°C. Once at the laboratory, samples were filtered through glass wool, split into 0.6-L aliquots, and amended with chemical surrogates (d_3-genistein and d_4-daidzein, Cambridge Isotopes, Andover, MA). Samples were then concentrated via solid phase extraction (SPE), cleaned with silica gel, and analyzed via liquid chromatography tandem mass spectrometry (LC-MS/MS) (described below).

Sample preparation for phytoestrogen analysis. SPE cartridges (6 mL 200 mg HLB Oasis cartridges, Waters, Milford, MA) were conditioned with two column volumes of acetone followed by two column volumes of ultrapure (Milli-Q, Millipore, Billerica, MA) water. Samples were loaded onto the SPE cartridge at a flow rate <10 mL/min. One column volume of a 1:8 MeOH:ultrapure water solution was then flushed through each cartridge to remove salts and polar organics. The SPE cartridges were vacuumed to dryness and immediately frozen at -20^{o}C. Two column volumes of acetone were used to elute phytoestrogens from the cartridges and were collected in conical sample vials. For

120 additional sample clean-up, the acetone was blown down to approximately 0.25 mL
121 using a gentle stream of nitrogen (UHP/Zero-grade). Silica gel columns were prepared
122 by placing a glass wool plug in a glass pipette then loading 1 gram of silica gel into the
123 pipette. Columns were conditioned with two column volumes of a 60:40
124 acetone:hexanes solution. Concentrated samples, followed by 6 mL of 60:40
125 acetone:hexanes solution were loaded onto the silica gel column without allowing the
126 column to run dry. Permeate was collected in a conical sample vial then blown down to
127 dryness under a gentle nitrogen stream (UHP/Zero-grade). The samples were then
128 redissolved in 100 µL of 70:30 ultrapure water:methanol, placed in silanized 300 µL
129 HPLC vials (ChromTech, Apple Valley, MN), and analyzed using LC-MS/MS. All
130 solvents used for sample preparation were HPLC-grade.
131 *High pressure liquid chromatography (HPLC) and liquid chromatography-mass*
132 *spectrometry (LC-MS).* HPLC and LC-MS were used to confirm the concentrations of
133 phytoestrogens in the minnow exposure experiments. These analytical methods are
134 described in detail in the supporting information.
135 *LC-MS/MS analysis of phytoestrogens.* Analyte separation was achieved on a
136 Waters NanoAcquity Ultra Performance Liquid Chromatography system equipped with a
137 Phenomenex Synergi 4u Polar-RP 80A column (150 x 0.55 mm 4µm). The following
138 optimized elution gradient, modified from Kang et al. [26], was applied at room
139 temperature with a flow rate of 10 µL/min: 80% A (20% B) at t=0 min, 16.2% A at t=17
140 min, 5% A at t=18 min, 5% A at t=23 min, 80% A at t=24 min, and 80% A t=38min
141 [Eluent A consisted of 95% 10 mM ammonium acetate and 5% acetonitrile, eluent B
142 consisted of 95% acetonitrile and 5% 10 mM ammonium acetate]. Sample injection

volume was 5 µL. Analyte detection was performed by means of negative ESI tandem mass spectrometry on a Thermo Finnigan TSQ Quantum Ultra MS. Limits of quantification (LOQs) were determined to be 1.43, 1.7, 2.05, 3.83, 2.01, and 2.31 µg/L for daidzein, genistein, formononetin, coumestrol, biochanin A, and zearalenone, respectively. Absolute recovery through SPE and silica gel cleanup was as follows: genistein 35% ± 6.4% (n=9), daidzein 64% ± 5.5% (n=9), coumestrol 22.5% ± 6.6% (n=9), formononetin 93% ± 7.0% (n=9), biochanin A 61% ± 5.3% (n=9), and zearalenone 87% ± 6.3% (n=9). Process blanks were analyzed three times during the experiment. Ultrapure water was collected in a 3.78-L glass container (the same bottle used for sample collection) and treated in the same manner as the environmental samples. No phytoestrogens were detected in the process blanks. Additional details of the method are described in Supporting Information and in Table S1.

Laboratory exposure experiments

To assess the biological effects of phytoestrogens at concentrations reflecting environmental occurrence, larval or adult fathead minnows were exposed for 21 days in a 50% daily static-renewal system or a flow-through system, respectively. We compared the potency of three phytoestrogens: genistein, daidzein, (TCI America, Portland, OR, 96% and 95%, respectively), and formononetin (Acros Organics, Geel, Belgium, 99%) singly and in mixture. Following exposure, survival, growth and predator avoidance performance were assessed in the larval fathead minnows, while adult fish were examined for morphological, physiological and behavioral alterations that may affect reproductive output.

Larval fathead minnow phytoestrogen exposures. Larval fathead minnows (<24 hrs old) were obtained from the US Environmental Protection Agency aquaculture facility in Cincinnati, OH. Upon arrival, larval minnows (n=25) were randomly assigned to one of three exposure beakers providing 75 larvae per treatment. We compared six treatment groups: water blank, ethanol carrier, daidzein, formononetin, genistein, and a mixture of all three phytoestrogens (each at 1,000 ng/L (nominally) singly or in mixture). Larvae were exposed for 21 days via daily 50% static renewals providing constant environmental conditions (water temperature 21± 2°C; 16:8 light:dark photoperiod). Fish were fed 1 mL of a live brine shrimp solution (Brine Shrimp Direct, Ogden, UT) twice daily following established US EPA culturing procedures [27]. Composite water samples (200 mL from each of the three beakers in a treatment) were taken every four days to confirm phytoestrogen concentrations by HPLC (Table S2). Samples were acidified in 1L amber bottles and kept at 4°C until analysis.

Following exposure, predator avoidance performance was assessed following previously published protocols [7,8]. Briefly, larvae were transferred individually to a testing arena to film their response to a vibrational stimulus mimicking an approaching predator. Larval fish were examined through cycling individuals by treatments (i.e. control, genistein, daidzein, formononetin, Mix-L, Mix-H, control, genistein, etc.). A total of ten fish from each beaker (n=30/treatment) were used for this analysis, with each larvae being filmed only once for the analysis. Larval body length (BL, mm), latency (time to response, msec), and escape velocity (BL/msec) were quantified. All data were digitized using morphometric software (NIH ImageJ).

187 *Adult fathead minnow phytoestrogen exposures.* Mature (approximately six
188 months old) fathead minnows were obtained from a laboratory fish supplier
189 (Environmental Consulting and Testing Inc., Superior, Wisconsin). Each treatment
190 contained four aquaria with randomized assignment of 10 males per 35-L aquarium (2
191 aquaria) or 10 females (2 aquaria). The seven treatments included the phytoestrogens
192 daidzein, genistein, and formononetin, singly at a nominal concentration of 1,000 ng/L
193 and mixtures of the three compounds at a low (Mix-L, all three phytoestrogens at 333
194 ng/L, nominally) and high concentration (Mix-H all three phytoestrogens at 1,000 ng/L,
195 nominally). A negative control (ethanol equivalent) served as baselines to compare
196 biological responses. Fish maintenance followed established protocols [27]. The flow-
197 through exposure system continuously pumped the appropriate amount of a
198 concentrated phytoestrogen stock solution into a temperature-adjusted flow of well
199 water supplied by a dedicated in-house well. The system provided flow rates of 200
200 mL/min per aquarium, allowing for approximately eight exchanges per day. All
201 environmental parameters during the exposure experiment were maintained constant as
202 follows: 21 ± 1°C; 5.5 ± 1.0 mg/L dissolved oxygen; 240 ppm $CaCO_3$ alkalinity; 0.95 ±
203 0.02 mS/cm conductivity; and 8.1 ± 0.1 pH. Abiotic parameters were monitored using a
204 YSI-556 unit and Hach 5-in-1 test strips. Ammonia was measured weekly using a Hach
205 (Loveland, CO) low-range test kit (mg/L). Water temperature was recorded using Hobo
206 data loggers (Onset Computer Corp., Bourne, MA) set to record temperature at 10-
207 minute intervals. Water samples were collected in 1-L bottles at weekly intervals
208 throughout the experiment and analyzed to calculate system loss. Samples were
209 acidified using 0.2 mL H_2SO_4 and stored at -4°C to prevent chemical degradation prior

to analysis. Water chemistry data, as measured on the LC-MS for the exposure experiments is provided in the Supporting Information (Table S3).

Post-exposure assessment of fathead minnows. Following the 21-day exposure, 10 male and 10 female fish from each treatment were sacrificed and dissected, with the remaining fish moved to the reproductive assay (see below). Mass (g) and total and standard length (mm) were taken prior to dissection to calculate body condition factor [(total mass/(total length^3))*100,000] [28]. Secondary sex characteristics for males were rated for color, breeding tubercle prominence, and dorsal pad thickness and summed for statistical analysis. Hepatic-somatic index (HSI=(liver mass/total mass)*100) and Gonadal-somatic index (GSI=(gonad mass/total mass)*100)) were calculated post dissection. After excision, liver and gonadal tissues were immediately weighed (g) and stored in 10% buffered formalin. Tissues were dehydrated using a Leica (Wetzlar, Germany) 1050 Automated Tissue Processor and embedded using a Tissue-Tek (Torrance, CA) Embedding Center. Tissues were sectioned using a Jung 2030 manual microtome to prepare slides to be stained using a Leica Autostainer XL (Haematoxylin and Eosin staining) after 24 hours. Livers were assessed for the prominence of vacuoles within hepatocytes using a severity scale (1-inconspicuous to 4-prominent vacuoles) following protocols established by the US EPA [29].

Whole blood was taken from the caudal region using heparinized capillary tubes. A competitive ELISA (polyclonal fathead minnow antibody) was used to quantify vitellogenin (VTG) concentrations by incorporating a species-validated anti-VTG antibody and purified VTG as standard following previously published protocols [30]. Standard curves were generated using Ascent software with eight standard

concentrations ranging from 0.075 to 4.8 µg/mL. The minimum detection limit was 3.75 µg/mL. For statistical purposes, samples below the lower detection limits were given a value of 1.875 µg/mL and above detection defined as 4,800 µg/mL.

The remaining fish were transferred as single-treatment pairs of one male and one female fish to 4-L spawning aquaria. Varying survival during the exposure and unequal quantities of male and female fish reduced the number of pairings per treatment that could be established to nine for the ethanol control and genistein exposures, eight for formononetin and Mix-L, seven for daidzein and six for Mix-H. Each aquarium contained a spawning tile constructed from a half section of 10-cm diameter PVC pipe. Spawning was recorded daily after afternoon feeding for two weeks. Spawning tiles with eggs were replaced while the tiles with eggs were placed into aerated 1-L beakers to monitor successful embryonic development (formation of eyespots) and hatching rates. Following previously published protocols [30], male nest defense was assessed three times in the week following phytoestrogen exposure. Briefly, a decoy minnow was lowered to the nest entrance to evoke male attacks. Latency, defined as time (s) to first attack (no response within five min was assigned a value of 300 s), and the number of attacks were enumerated for 60 s after initial contact. A total aggression index (TAI) was calculated by dividing the number of attacks (multiplied by a factor of 10 to equally weigh parameters) by male latency to first attack. Testing order was randomized and observations were averaged across testing events for statistical analysis [7,8].

Statistical Analysis

Data were tested for normality using a Kolgomorov-Smirnov test. For the larval exposure experiment, beakers within treatments were compared by ANOVA prior to data being combined, as no beaker-specific differences were found. Normally distributed data were compared using ANOVAs followed by a Dunn's post test. Plasma VTG concentrations were log10 transformed prior to statistical analysis by ANOVA. The nonparametric Kruskal-Wallis test was used to compare responses in data with non-parametric distributions. Larval survival was compared relative to ethanol carrier controls via Fisher's exact test (2×2 contingency table). (Prism 6.0 statistical package, GraphPad Software Inc., Oxnard, CA). The Friedman test was used to compare daily egg production between treatments, followed by a Dunn's post test. Significance for all tests was pre-established at a $p < 0.05$ level.

RESULTS

Occurrence of Phytoestrogens in the Environment

The analysis of surface water samples showed that some phytoestrogens were present in the low nanogram per liter range in the human-impacted samples (Lake Vadnais and the Metro Plant effluent channel) whereas none of the monitored phytoestrogens were detected in relatively pristine Straight Lake (n=12). Genistein, daidzein, and formononetin were detected in 5, 4, and 7 out of 12 total samples, respectively, taken at Lake Vadnais, and in 4, 3, and 1 out of 7 total samples, respectively, taken in the Metro Plant effluent channel. When detected, the average concentrations were 1.4 ± 0.5 ng/L, 1.6 ± 0.7 ng/L, and 1.1 ± 0.2 ng/L for genistein, daidzein, and formononetin, respectively, in Lake Vadnais, and 1.6 ± 0.4 ng/L, 1.8 ± 1.3

278 ng/L, and 2.0 ng/L for genistein, daidzein, and formononetin, respectively, in the Metro
279 Plant effluent channel. Biochanin A was also detected twice (1.1 and 0.9 ng/L), but only
280 in Lake Vadnais. Zearalenone and coumestrol were never detected. In addition, no
281 clear temporal trends with respect to phytoestrogen concentration were evident from the
282 data (data not shown).

283 Similarly, even downstream of suspected anthropogenic phytoestrogen sources
284 on the Minnesota River and Okabena Creek, the phytoestrogens monitored in this study
285 were only detected in the low nanogram per liter range (Figure S1). Surface water
286 samples collected from the Minnesota River in June contained all phytoestrogens
287 monitored with the exception of coumestrol (Figure S1A). Only one of the samples from
288 the Minnesota River collected in November (the effluent sample from the Mankato
289 Wastewater Treatment Plant) contained detectable phytoestrogens (daidzein and
290 formononetin, Figure S1B). Samples from Okabena Creek showed a similar pattern of
291 low phytoestrogen presence, with only genistein and daidzein detected in the low
292 nanogram per liter range (Figure S2). These results suggest that the sampled
293 phytoestrogens attenuate rapidly in the environment, likely reducing fish exposure risk
294 to those areas immediately downgradient of more concentrated phytoestrogen
295 discharge points [*e.g.*, 23].

296 *Larval Minnow Exposure to Phytoestrogens*

297 Phytoestrogen exposure had dramatic effects on larval survival. The Fisher's
298 Exact Test revealed significant declines in survival for genistein ($p<0.001$), formononetin
299 ($p<0.001$), and mixture ($p<0.001$) treatments relative to the ethanol control (Figure 1A).
300 No difference was found when comparing survival of daidzein-exposed larvae with that

301 in the ethanol carrier control (p=0.12). A compound at the daidzein retention time on the
302 HPLC, either daidzein itself or a co-eluting compound produced in this highly
303 biologically active system, was detected in all samples (including the ethanol control)
304 (Table S1). Because no effect was seen on larval minnows with daidzein exposure, this
305 was deemed to be irrelevant. Despite the decrease in survival, those larvae that did
306 survive were of similar length (Figure 1B) and had reaction times to a threatening
307 stimulus that were comparable to those of control larvae (Figure 1C). In addition,
308 escape velocity was not affected by exposure to single phytoestrogens or a mixture of
309 phytoestrogens (Figure 1D).

310 *Adult minnow exposure to phytoestrogens*

311 Biological effects on adult minnows were generally subtle, with exposures to
312 genistein, daidzein, formononetin and mixtures of the three causing no statistically
313 significant (with 95% confidence) effects on body condition factor, gonadosomatic index,
314 hepatosomatic index, liver vacuolization, or plasma VTG concentrations in male (Table
315 1) or female fathead minnows (Table S4). Analysis of expression of secondary sex
316 characteristics and nest defense behavior did not reveal statistically significant
317 differences in total aggression assay among males across treatments (Table 1).
318 Nevertheless, non-statistically significant feminizing trends were consistently observed
319 across all of the experiments during phytoestrogen exposure as suggested by the
320 aggregated ranking of all measured dependent variables (Table 1). Despite the
321 generally subtle effects of phytoestrogen exposure on adult minnows, egg production
322 was found to be significantly greater in adult female minnows that were exposed to
323 daidzein as compared to those in the ethanol control or any other treatment ($p<0.001$,

Figure 2).

DISCUSSION

Phytoestrogens are discharged to the environment from anthropogenic and natural sources and can reach concentrations >1000 ng/L in municipal wastewater treatment plant effluent [*e.g.*, 23]. Of the six phytoestrogen species measured in this study, four (genistein, daidzein, formononetin, and biochanin A) were detected in human-impacted surface waters (Lake Vadnais, the effluent channel of the Metro Plant, the Minnesota River, and Okabena Creek), but not in a relatively pristine surface water (Straight Lake), indicating that non-anthropogenic inputs are likely to be irrelevant to the health of aquatic organisms. Our observation of periodic phytoestrogen presence at low concentrations in human-impacted water is similar to observations made in the literature [21,22,25,26,31,32], where low phytoestrogen concentrations were also measured downstream of likely anthropogenic sources. No obvious temporal or spatial trends were observed with respect to detection or the concentration of the phytoestrogens detected.

With respect to the minnow exposure data, two observations can be made: (1) larval minnow survival is diminished when exposed to genistein, formononetin or a mixture of genistein, daidzein, and formononetin (Figure 1), and (2) adult minnows are minimally impacted by genistein, daidzein, and formononetin exposure except with respect to egg production, where a stimulatory effect of daidzein exposure was observed (Figure 2). With respect to larval fish, numerous studies suggest fish may be susceptible to contaminants during early ontogenetic stages [33,34] and although

previous research has demonstrated estrogenic impairment to escape performance in larval fish with 17β-estradiol exposure [7], the exposure of developing larvae to phytoestrogens appears to only decrease survival. With respect to adult minnows, our experiments suggest that the threshold for biological changes, particularly in males, occurs at higher concentrations. This is despite the previously demonstrated estrogenic nature of phytoestrogens [11,35,36]. Indeed, parallel to the nest defense total aggression index seen in this study, Clotfelter and Rodriguez [11] reported no significant declines in latency by males below 1,000 µg/mL. A trend of the subtle feminization of adult minnows, including increased plasma VTG and decreased secondary sex characteristics, was observed with phytoestrogen exposure (Table 1). These finding suggest that fish exposed at a higher concentrations, for a longer period, or during a different developmental stage (for example sexual differentiation) may exhibit a more pronounced estrogenic response.

The lack of any effects observed in mature female fathead minnows (other than changes to fecundity) is not surprising as the naturally much higher plasma concentrations of estradiol in female fish would likely buffer any effect of these weakly estrogenic phytoestrogens.

This research indicates that genistein, daidzein, and formononetin are rapidly attenuated in the environment and they are unlikely to cause widespread ecological harm in surface waters, except in the cases of female minnow egg production and larval survival. This points to a need for perhaps more treatment of anthropogenic phytoestrogen sources during critical developmental periods (e.g., larval development) and in those locations where high concentrations of phytoestrogens are likely, such as

industrial discharges or discharges from municipal wastewater treatment plants that receive waste from particular industries [4,19,20,23]. Furthermore, effluent discharge sites often attract fish due to higher water temperatures and greater nutrient supplies and may result in fish being exposed continuously to phytoestrogens at higher concentrations than can be expected below the mixing zone. Exposure to "pseudo-persistent" endocrine active compounds has been identified in several studies as a common "worst-case" scenario for the exposure of aquatic organisms [37,38] and could occur with phytoestrogens. In addition, little is known about the degradation products of phytoestrogens [e.g., 35], and although the parent compounds do appear to attenuate readily, degradation products may be formed with estrogenic or androgenic character (Kelly et al., submitted). Finally, phytoestrogens are likely to sorb to sediment [24,39,40], and could build up in sediment, particularly over cold-temperature seasons when environmental attenuation would be expected to be slower (Kelly et al., submitted). Based on the consistent, yet non-statistically significant estrogenic trends observed in our experiments (Table 1), it is possible that sorbed phytoestrogens could impact wild minnow populations with particle ingestion or during egg production and larval life stages.

Although a step in the right direction, our study only focused on a specific time period of exposure, during breeding and larval development. Chronic exposure could cause different results or even biological compensation to impairment. Future research examining complexities of seasonal variation in environmental estrogen discharge, compound attenuation, and seasonal fish vulnerability will help provide relevant ecosystem-based risk assessment.

ACKNOWLEDGEMENTS

Funding for this project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR). We thank the undergraduate research students at St. Cloud State University who assisted in the fish dissections. We also thank Peter Villalta at the University of Minnesota Masonic Cancer Center Mass Spectrometry Facility for assistance with the phytoestrogen quantification.

REFERENCES

1. Falconer IR, Chapman HF, Moore MR, Ranmuthugala G. 2006. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. *Environ Toxicol* 21:181–191.

2. Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley, GT, Foster PM, Gray CL, Gray LE. 2008. Fifteen years after 'Wingspread' — environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol Sci* 105:235–259.

3. Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty GC, Sumpter JP, Tyler CR. 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* 66:272–281.

4. Larsson DG, Forlin L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. *Environ Health Perspect* 110:739-742.

5. Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maak G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ Health Perspect* 112:1725-1733.

6. Saaristo M, Craft JA, Lehtonen KK, Björk H, Lindström K. 2009. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinyl estradiol, an endocrine disruptor. *Horm Behav* 55:530-537.

7. McGee MR, Julius ML, Vajda AM, Norris DO, Barber LB, Schoenfuss HL. 2009. Predator avoidance performance of larval fathead minnows (*Pimephales promelas)* following short-term exposure to estrogen mixtures. *Aquat Toxicol* 91:355-361.

8. Painter MR, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, Furlong ET, Schultz MM, Schoenfuss HL. 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows *(Pimephales promelas)*. *Environ Toxicol Chem* 28:2677-2684.

9. Swain DP, Lindsey CC. 1984. Selective predation for vertebral number of young sticklebacks, *Gasterosteousa culeatus*. *Can J Fish Aquat Sci* 8:1231-1233.

10. Burroughs CD. 1995. Long-term reproductive tract alterations in female mice

435 treated neonatally with coumestrol. *Proc Soc Exp Biol Med* 208:78-81.

436 11. Clotfelter E, Rodriguez, A. 2006. Behavioral changes in fish exposed to
437 phytoestrogens. *Environ Pollut* 144:833-839.

438 12. Maclatchy DL, Vanderkraak GJ. 1995. The phytoestrogen beta-sitosterol alters
439 the reproductive endocrine status of goldfish. *Toxicol Appl Pharmacol* 134:305-
440 312.

441 13. Pace V, Carbone K, Spirito F, Lacurto M, Terzano M, Verna M, Vincenti F,
442 Settineri D. 2006. The effects of subterranean clover phytoestrogens on sheep
443 growth, reproduction and carcass characteristics. *Meat Sci* 74:616-622.

444 14. Tremblay L, Van Der Kraak G. 1999. Comparison between the effects of
445 phytosterols β-sitosterol and pulp and paper mill effluents on sexually immature
446 rainbow trout. *Environ Toxicol Chem* 18:329-336.

447 15. Jefferson W, Padilla-Banks E, Newbold R. 2007. Disruption of the developing
448 female reproductive system by phytoestrogens: Genistein as an example. *Mol*
449 *Nutr Food Res* 51:832-844.

450 16. Pelissero C, Menn FL, Kaushick S. 1991. Estrogenic effect of dietary soya bean
451 meal on vitellogenisis in cultured Siberian sturgeon *Acipenser baeri*. *Gen Comp*
452 *Endocrinol* 83:447-457.

453 17. Markaverich BM, Webb B, Densmore CL, Gregory RR. 1995. Effects of
454 coumestrol on estrogen receptor function and uterine growth in ovariectomized
455 rats. *Environ Health Perspect* 103:574-581.

18. Kiparissis Y, Balch G, Metcalfe T, Metcalfe C. 2003. Effects of isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*). *Environ Health Perspect* 111:1158-1163.

19. Pollock MS, Dubé MG, Schryer R. 2010. Investigating the link between pulp mill effluent and endocrine disruption: attempts to explain the presence of intersex fish in the Wabigoon River Ontario, Canada. *Environ Toxicol Chem* 29:952-965.

20. Theodorakis CW, Lee K, Adams M, Law CA. 2006. Evidence of altered gene flow, mutation rate, and genetic diversity in redbreasted sunfish from a pulp-mill-contaminated river. *Environ Sci Technol* 40:377-386.

21. Bacaloni A, Cavaliere C, Faberi A, Foglia A, Samperi R, Lagana A. 2005. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants, *Analytica Chimica Acta* 531:229-237.

22. Kolpin D, Furlong E, Meyer M, Thurman M, Zaugg S, Barber L, Buxton H. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol* 36:1202-1211.

23. Lundgren MS, Novak PJ. 2009. Quantification of phytoestrogens in industrial waste streams. *Environ Toxicol Chem* 28:2318–2323.

24. Hartmann N, Erbs M, Wettstein F, Horger C, Vogelgsang S, Forrer H, Schwarzenbach R, Bucheli T. 2008. Environmental exposure to estrogenic and other myco- and phototoxins. *Chimia* 62:364-367.

25. Kolpin D, Hoerger C, Meyer M, Wettstein F, Hubbard L, Bucheli T. 2010. Phytoestrogens and mycotoxins in Iowa streams: an examination of underinvestigated compounds in agricultural basins. *J Environ Qual* 39:2089-2099.

26. Kang J, Price W. 2009. Occurrence of phytoestrogens in municipal wastewater and surface waters. *J Environ Monit* 11:1477-1483.

27. Denny JS. 1987. Guidelines for the culture of fathead minnows *Pimephales promelas* for the use in toxicity tests. EPA/600/3-87/001.

28. Fulton TW. 1904. The rate of growth of fishes. Fisheries Board of Scotland, Annual Report 22(3):141-241.

29. Wolf JC. 2008 Histology and histopathology guidelines for phase 1V if OECD fish screening assay for EDCs. WA #04-14; US Environmental Protection Agency EPL Project No. 481-017, Washington, DC.

30. Dammann AA, Shappell NW, Bartell SE, Schoenfuss HL. 2011.Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, Pimephales promelas. *Aquat Toxicol* 105:559-568.

31. Hoerger C, Wettstein F, Hungerbuhler K, Bucheli T. 2009. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ Sci Technol* 43;6151-6157.

32. Lagana A, Bacaloni A, De Leva I, Faberi A, Fago G, Marino A. 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals

in sewage treatment plants and natural waters. *Analytica Chimica Acta* 501:79-88.

33. Koger CS, Teh SJ, Hinton DE. 2000. Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17β-estradiol or testosterone. *Mar Environ Res* 50:201-206.

34. Guillette Jr LJ, Crain DA, Rooney AA, Pickford DB. 1995. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ Health Perspect* 103:157-164.

35. Ellis R, Van Den Heuvel M, Bandelj E, Smith M, McCarthy L, Stuthridge T, Dietrich D. 2003. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ Tox Chem* 22:1448-1456.

36. Hewitt LM, Kovacs TG, Dube MG, MacLatchy DL, Martel PH, McMaster ME, Paice MG, Parrott JL, van den Heuvel MR, Van Der Kraak GJ. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: roles of individual compounds and mill operating conditions. *Environ Toxicol Chem* 3:682-697.

37. Barber LB, Brown GK, Nettesheim TG, Murphy EW, Bartell SE, Schoenfuss HL. 2011. Effects of biologically-active chemical mixtures on fish in a wastewater-impacted urban stream. *Sci Total Environ* 409:4720-4728.

38. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care pro- ducts in the environment: agents of subtle change? *Environ Health Perspect* 107(Suppl 6):907–38.

519 39. Eaton A, Clesceri L, Greenberg A. 1995. Standard Methods for Examination of
520 Water and Wastewater 19th Edition. American Public Health Association,
521 Washington, DC.

522 40. Schenzel J, Goss K, Schwarzenbach R, Bucheli T, Droge S. 2012.
523 Experimentally determined soil organic matter-water sorption coefficients for
524 different classes of natural toxins and comparison with estimated numbers.
525 *Environ Sci Technol* 46:6118-6126.

526

527

528

Reference in Review (attached as supplement):

Kelly MM, Fleischhacker N, Rearick DC, Arnold WA, Schoenfuss HL, Novak PJ. In review. Microbial degradation of phytoestrogens and the response of fathead minnows to degraded exposure.

Table 1. Mean ± standard error of body condition factor (BCF), gonadosomatic index (GSI), hepatosomatic index (HSI), liver hepatocyte vacuolization (Vacuole – not ranked), sum of secondary sex characteristics (SSC), plasma vitellogenin concentrations (VTG), and total aggression index (TAI) by treatment in male fathead minnows. Ranking and aggregation of biological trends observed in male fathead minnows across treatments are listed below the measured value in bold Italic font. Similar values are given the mean rank. Lowest rank for any dependent variable suggests least feminization (based on the direction in which values would be observed in female fathead minnows).

	BCF	**GSI**	**HSI**	**Vacuole**	**SSC**	**VTG (ug/mL)**	**TAI**	***Aggregate rank***
EtOH (n=14)	1.08±0.04	0.92±0.09	1.2±0.17	3.01±0.26	6.08±0.4	861±195	31.75±12.8	
	3	***1.5***	***3***		***1***	***3***	***2***	***13.5***
Genistein (11)	1.05±0.03	0.92±0.16	1.35±0.18	3.18±0.26	5.46±0.64	1311±479	14.08±5.9	
	2	**1.5**	***5***		***3***	***5***	***4***	***20.5***
Daidzein (12)	1.01±0.05	1.84±0.54	0.99±0.14	3.27±0.27	4.5±0.71	726±420	37.07±20.7	
	1	***5***	***1***		***2***	**2**	***1***	***12***
Formononetin	1.12±0.03	1.27±0.26	1.3±0.15	3.08±0.15	5.41±0.42	479±143	13.14±5.1	
(13)	***5***	***4***	***4***		***4***	***1***	***5***	***23***
Mix-Low (12)	1.1±0.03	1.04±0.18	1.06±0.15	2.92±0.26	4.3±0.37	1477±584	19.7±18.1	
	4	***2***	***2***		***6***	***6***	***3***	***23***
Mix-High (11)	1.16±0.05	1.14±0.19	1.68±0.21	3±0.23	5.09±0.64	875±518	7.32±4.3	
	6	***3***	***6***		***5***	***4***	***6***	***30***

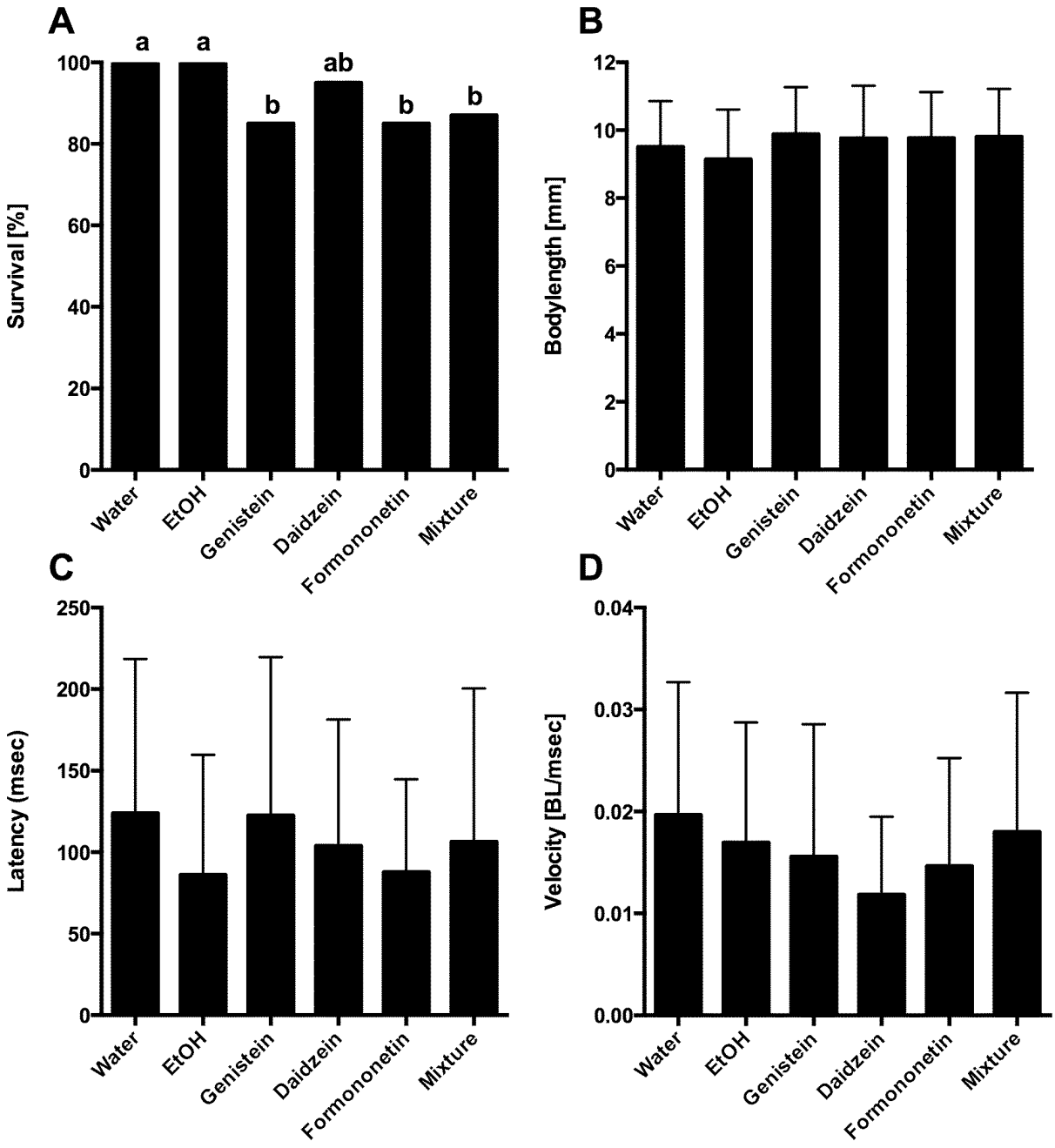
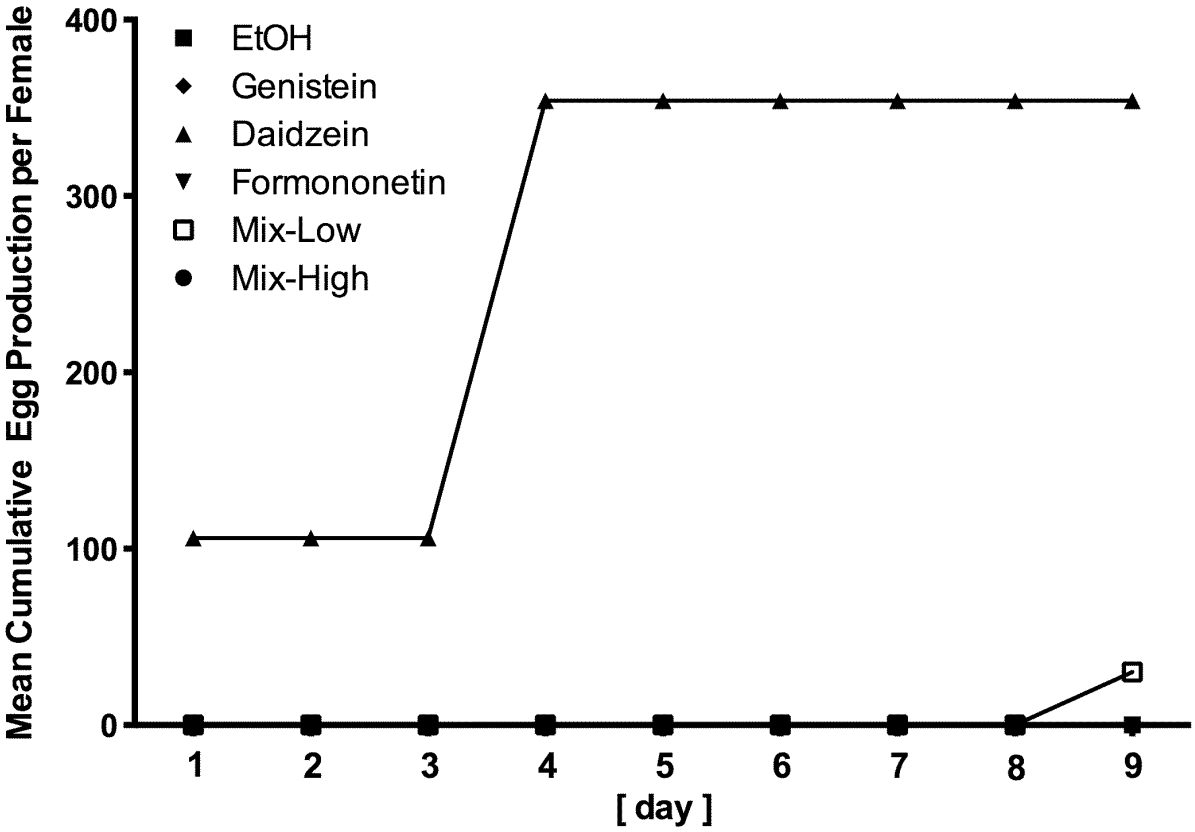


Figure 1. Larval survival (A), growth (B), latency (C) and escape velocity (D) after 21 day exposure to phytoestrogen singly or in mixture. Small caps in (A) indicate significant differences in survival among treatments (Fisher Exact Test, $p<0.05$); sample size in (B-D) = 30/ treatment.



547

548

549 **Figure 2.** Mean cumulative egg production for female fathead minnows exposed 21-
550 days to phytoestrogens. Egg production was monitored daily for nine days.

551

552

PHYTOESTROGENS IN THE ENVIRONMENT: I. OCCURRENCE AND EXPOSURE EFFECTS ON FATHEAD MINNOWS

DANIEL C. REARICK †, NATHAN FLEISCHHACKER ‡, MEGAN M. KELLY §, WILLIAM A. ARNOLD ‡§ , PAIGE J. NOVAK ‡ §, AND HEIKO L. SCHOENFUSS †*

† Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

METHODS

HPLC analysis. All HPLC analyses were performed with an Agilent 1200 series HPLC system with photodiode array detection. The LC was equipped with an Ascentis RP-Amide column (15 cm x 4.6 mm, 5 µm, Supelco). A double solvent system with internal buffer was used: solvent A consisted of 10 mM ammonium acetate in 90% pure water (Milli-Q, Millipore) and 10% HPLC-grade acetonitrile adjusted to pH 5 with glacial acetic acid; solvent B was 100% HPLC-grade acetonitrile. The flow rate for the mobile phase was 1 mL/min and was operated isocratically with 40% solvent A, 60% solvent B. Genistein was detected at 259 nm, daidzein was detected at 249 nm, and estradiol was detected at 230 nm. The limits of quantification (LOQ) for genistein, daidzein, and estradiol on the HPLC were 19 µg/L, 50 µg/L, and 8 µg/L, respectively.

LC-MS analysis. A Hewlett-Packard 1050 model liquid chromatograph equipped with an Agilent 1100 Mass Spectrometer Detector and Agilent ChemStation software was used to analyze samples in selected ion mode. The same column and two-solvent system used in HPLC was used for LC separation. The following elution gradient was used: 60% A (40% B) at t=0 min, linear addition of solvent B to 45% by t=25 min, followed by a 5 min flush of 100% B, then ending in a 5 min equalization of 45% solvent B. The LC effluent was fed directly into the mass spectrometer with electrospray ionization source operated at 300^{o}C in negative ion mode. Nitrogen was used as the drying and nebulizing gas and a fragment voltage of 70 mV was kept constant throughout the run. One scan window was used to identify genistein (269 m/z, 15 min), formononetin (267 m/z, 12.5 min), daidzein (253 m/z, 6.5 min), deuterated daidzein (256 m/z, 6.5 min) and deuterated genistein (272 m/z, 15 min). Peak area response

associated with each analyte was normalized by surrogate recovery to compensate for variation in machine performance and variable SPE recovery through the extraction process. For the LC-MS method the LOQ was 4.43 µg/L for genistein, 3.53 µg/L for daidzein and 2.79 µg/L for formononetin. The absolute recovery for the SPE process as determined in ultrapure water was 30.0% ± 1.1%, 41.1% ± 7.7%, and 30.4% ± 3.8% for daidzein, formononetin, and genistein, respectively.

LC-MS/MS analysis of phytoestrogens

Analyte separation and detection was performed as described in the manuscript. Interface parameters for the LC-MS/MS system were as follows: Capillary temperature 300^{o}C, skimmer offset -10, spray voltage 3000 V, and sheath gas 38. The collision cell gas (Ar, 99.999%) pressure was 1.5 mTorr, Detection of the phytoestrogens was performed using the mass transitions specified in Table S1. Analyte presence was confirmed based on a comparison of fragmentation ratios seen in standards, as well as elution time. The analytes were quantified using external calibration curves using standards in ultrapure water. To test recovery, 0.6 L of ultrapure water was amended with genistein, daidzein, coumestrol, biochanin A, formononetin, and zearalenone three different times at three different concentrations (0.5, 1, 10 ng/L). The spiked samples were treated in the same manner as the environmental samples, and recoveries were determined by comparing the quantity of compound added to the sample and subsequently measured by the LC-MS/MS. As stated in the manuscript, recovery through SPE and silica gel cleanup was not concentration-dependent, with absolute recoveries as follows: genistein 35% ± 6.4% (n=9), daidzein 64% ± 5.5% (n=9),

614 coumestrol 22.5% ± 6.6% (n=9), formononetin 93% ± 7.0% (n=9), biochanin A 61% ±
615 5.3% (n=9), and zearalenone 87% ± 6.3% (n=9).

616

617

618

619 **Table S1.** Optimized instrumental parameters for phytoestrogens and corresponding
620 surrogates

Compound	**Scan Event**	**Retention Time (min)**	**Precursor Ion (m/z)**	**Fragments** [1]	**Collision Energies (eV)**
Genistein	4	13.17	269.19	180, 159, **133**	32
D_3-genistein	4	13.17	272.19	183, **134**	32
Daidzein	1	11.17	253.19	223, **208,** 132	40
D_4-daidzein	1	11.16	256.19	226, **211**, 135	40
Formononetin	2	13.31	267.19	**182**, 166	45
Coumestrol	5	14.45	267.19	**252,** 223	25, 35
Biochanin A	3	16.49	282.19	268, **239**, 211	30, 35, 40
Zearalenone	2	16.43	317.19	**317**	25

621 [1] Bold fragments were used for quantification.

Table S2. Confirmatory water chemistry for the larval minnow phytoestrogen exposure experiment (mean ± st. err.; n=5; Note: n=1 for the ethanol control)

Treatment	Genistein	Daidzein	Formononetin
EtOH Control	10 ng/L	120 ng/L	70 ng/L
Genistein	308 ± 291 ng/L	244 ± 262 ng/L	38 ± 35 ng/L
Daidzein	18 ± 27 ng/L	676 ± 348 ng/L	106 ± 221 ng/L
Formononetin	22 ± 30 ng/L	502 ± 739 ng/L	414 ± 274 ng/L
Mixture	250 ± 149ng/L	812 ± 352ng/L	294 ± 192ng/L

Note: the daidzein detection in the ethanol control and in all other treatments not receiving daidzein was thought to be a co-eluting compound as a result of biological activity in the system. No response was observed in the daidzein exposed larvae or in the ethanol control; therefore, the presence of daidzein or a co-eluting compound in these samples was deemed to be unimportant.

Table S3. Confirmatory water chemistry for the adult minnow phytoestrogen exposure experiment (mean ± st. err.; n=3); nd is non-detect.

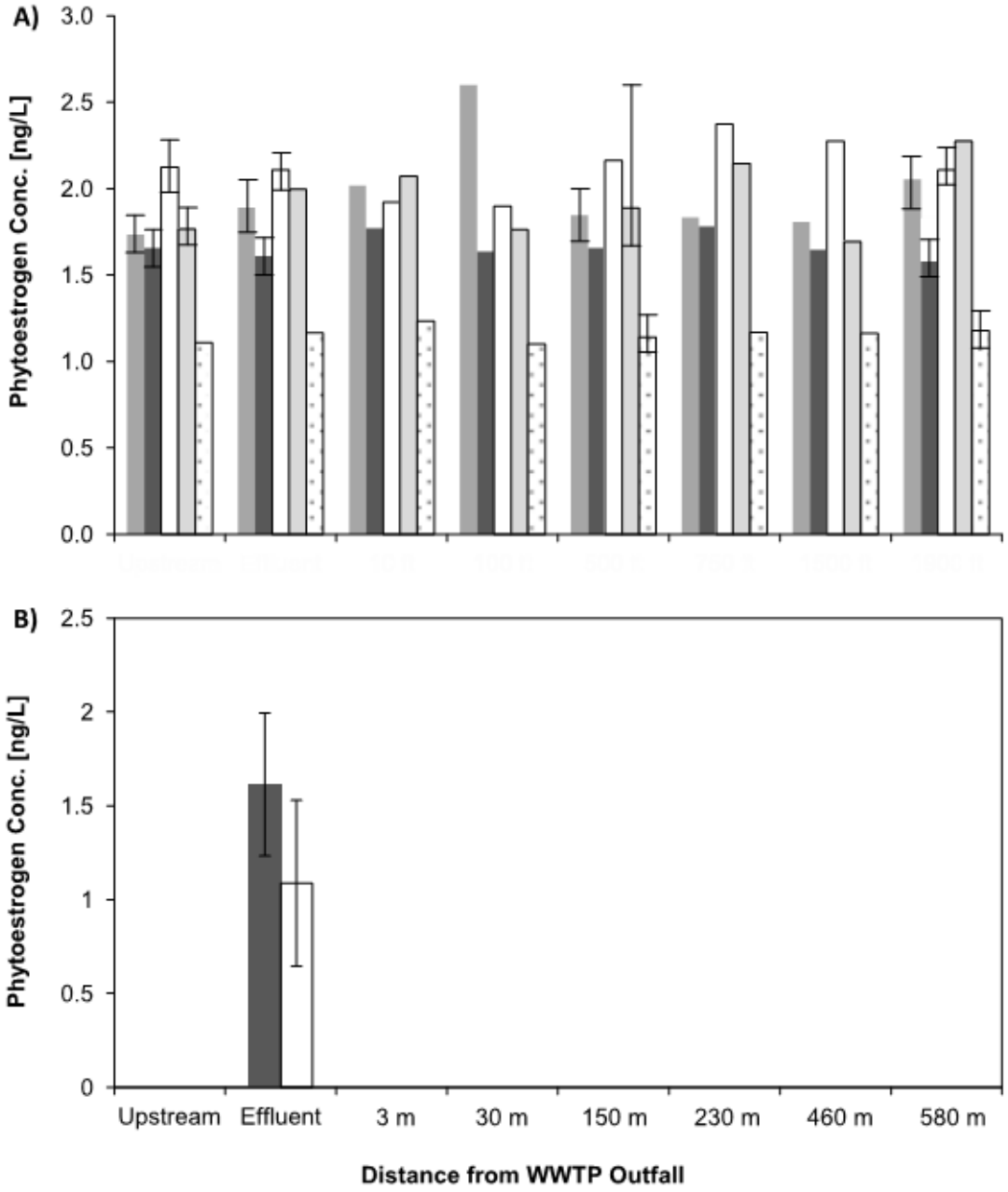
Treatment	Genistein	Daidzein	Formononetin
EtOH Control	nd	nd	nd
Genistein	440±170 ng/L	nd	nd
Daidzein	nd	1200±540 ng/L	nd
Formononetin	nd	nd	590±110 ng/L
Mix Low	160±28ng/L	280±120ng/L	380±94ng/L
Mix High	490±66ng/L	940±230ng/L	620±44ng/L

634 **Table S4.** Mean ± standard error of body condition factor (BCF), gonadosomatic index (GSI), hepatosomatic index (HSI),
635 liver hepatocyte vacuolization (Vacuole), and plasma vitellogenin concentrations (VTG) by treatment in female fathead
636 minnows.

	BCF	GSI	HSI	Vacuole	VTG (ug/mL)
EtOH (n=14)	1.06±0.06	7.56±1.22	1.64±0.22	2.67±0.37	1049±313
Genistein (11)	1.03±0.05	9±2.04	1.53±0.47	3±0.26	481±284
Daidzein (12)	1.08±0.22	9.4±1.93	1.52±0.15	3.11±0.39	386±103
Formononetin (13)	1.22±0.22	13.13±2.3	1.74±0.29	3.44±0.29	258±84
Mix-Low (12)	1±0.06	5.86±1.11	1.04±0.16	2.92±0.29	830±505
Mix-High (11)	0.98±0.04	7.45±1.63	1.01±0.12	2.67±0.33	1956±859

637

638



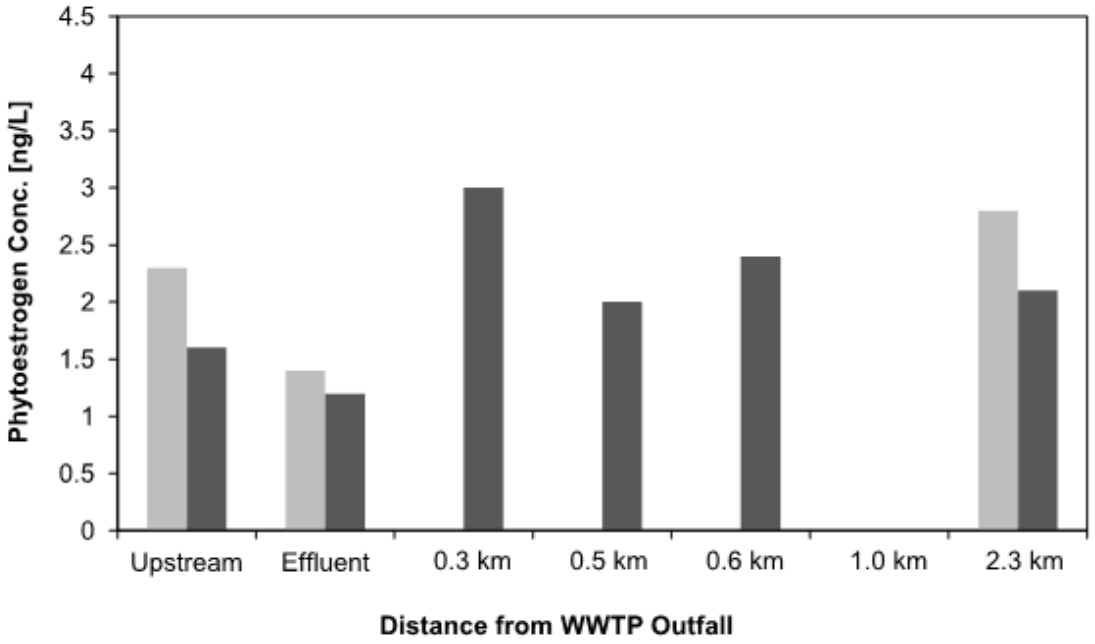
639

640 **Figure S1.** Concentrations of genistein (▫) daidzein (■), formononetin (□), biochanin A
641 (▫), and zearalenone (⚀) in water samples taken from the city of Mankato (MN) WWTP
642 effluent and the Minnesota River upstream and downstream of the WWTP effluent.
643 Distances are as measured downstream from the WWTP effluent discharge. Panel (A)
644 shows results from samples taken on June 20, 2011 and panel (B) shows results from

645 samples taken on November 8, 2011. Samples upstream of the effluent, the effluent
646 itself, and 150 and 580 m downstream of the effluent were collected in triplicate. Error
647 bars represent the standard deviation of these triplicate samples. Direct sampling of the
648 WWTP effluent was impossible on November 8, 2011 as a result of high water levels in
649 the Minnesota River.

650

651



652

653 **Figure S2:** Concentrations of genistein (▫) and daidzein (■) in water samples taken from the
654 city of Brewster (MN) WWTP effluent and Okabena Creek upstream and downstream of the
655 WWTP effluent on June 6, 2012. Distances are as measured downstream from the WWTP
656 effluent discharge. Samples upstream of the effluent, the effluent itself, and 2.3 km downstream
657 of the effluent were collected in triplicate. Error bars represent the standard deviation of these
658 triplicate samples.