Running Head: biodegradation of phytoestrogens and biological effects

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

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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.

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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

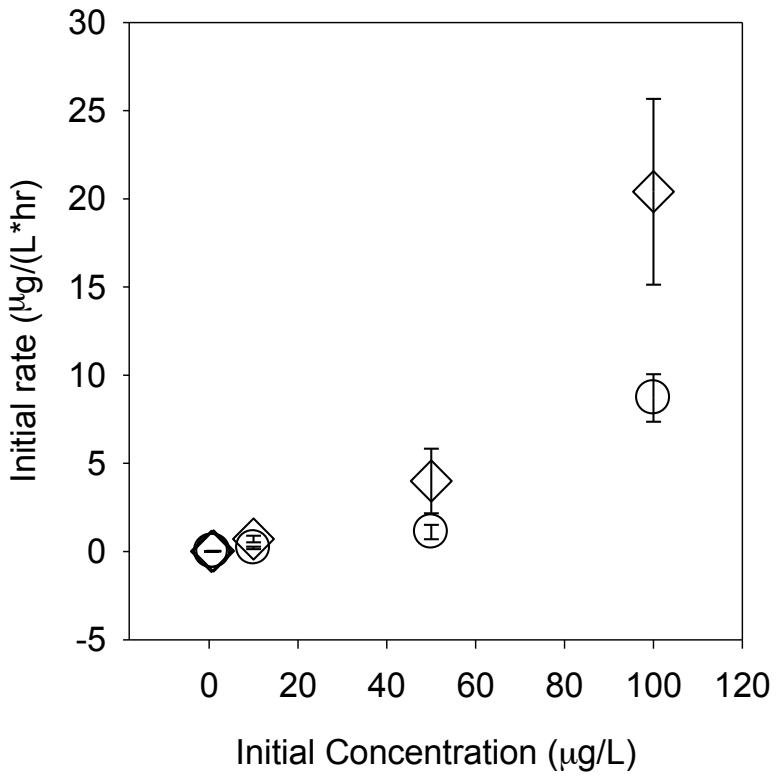

Figure 1. Initial degradation rates of genistein (diamonds) and daidzein (circles) (given as the first order rate constant multiplied by initial concentration) at different initial concentrations in Mississippi River water.

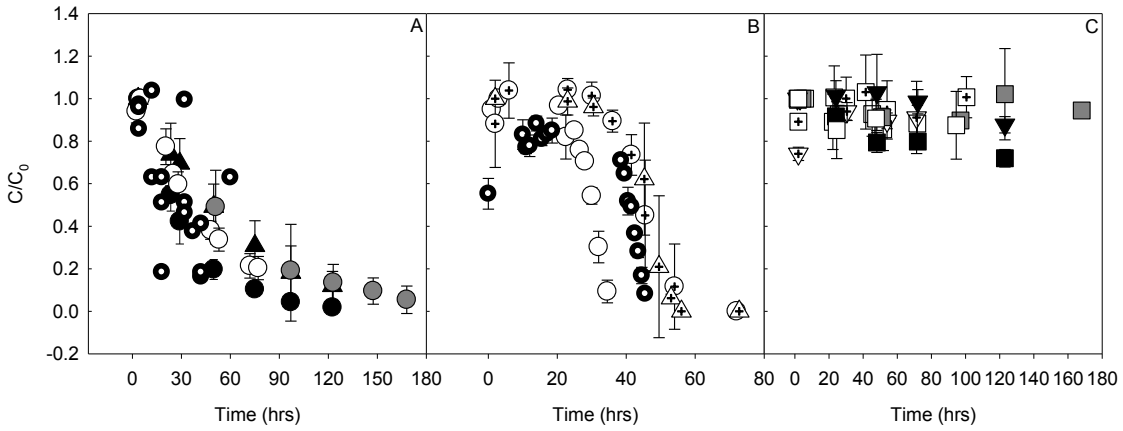

Figure 2. Degradation of genistein at an initial concentration of 0.5 µg/L (A) or 100 µg/L (B) in water collected on November 8, 2011 (cross-hairs), May 14, 2012 (white), June 6, 2012 (black), and June 26, 2013 (rings) from the Mississippi River (rings experimental, controls in SI) Okabena Creek (up 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.

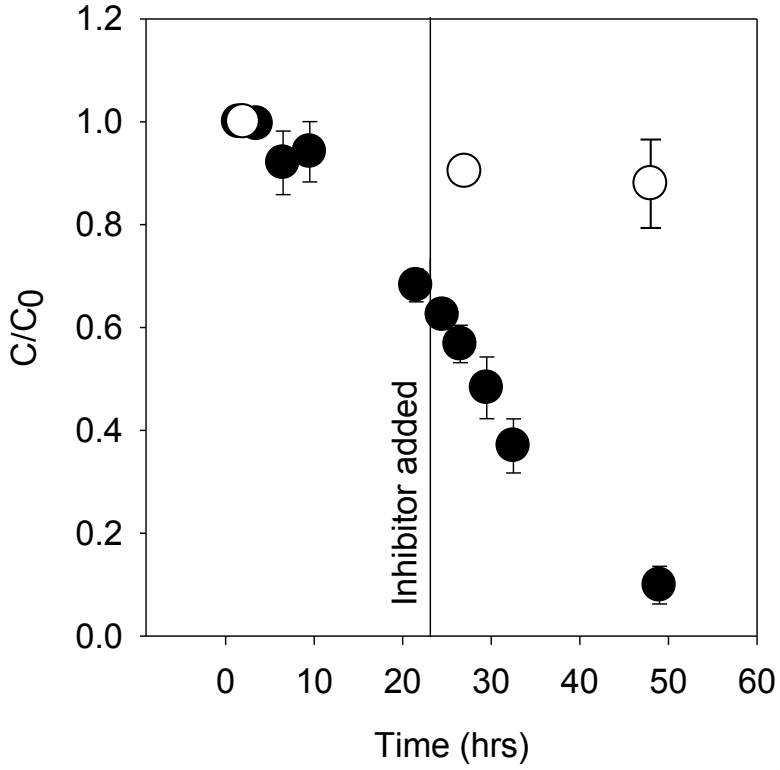

Figure 3. Degradation of genistein in an enriched nitrifying culture (black) and a killed control of the same culture (white) before and after the addition of allylthiourea, an inhibitor of ammonia oxidation.

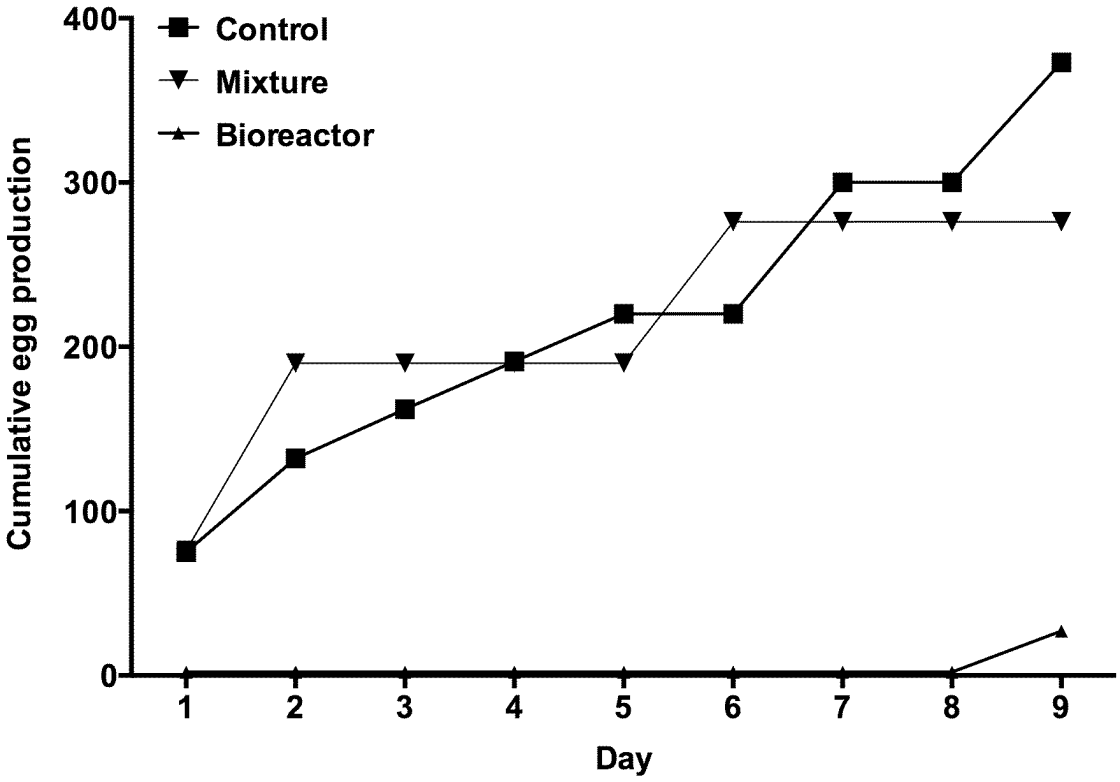

Figure 4. Cumulative egg production per treatment montiored for nine consecutive days.

SUPPORTING INFORMATION

For

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

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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.

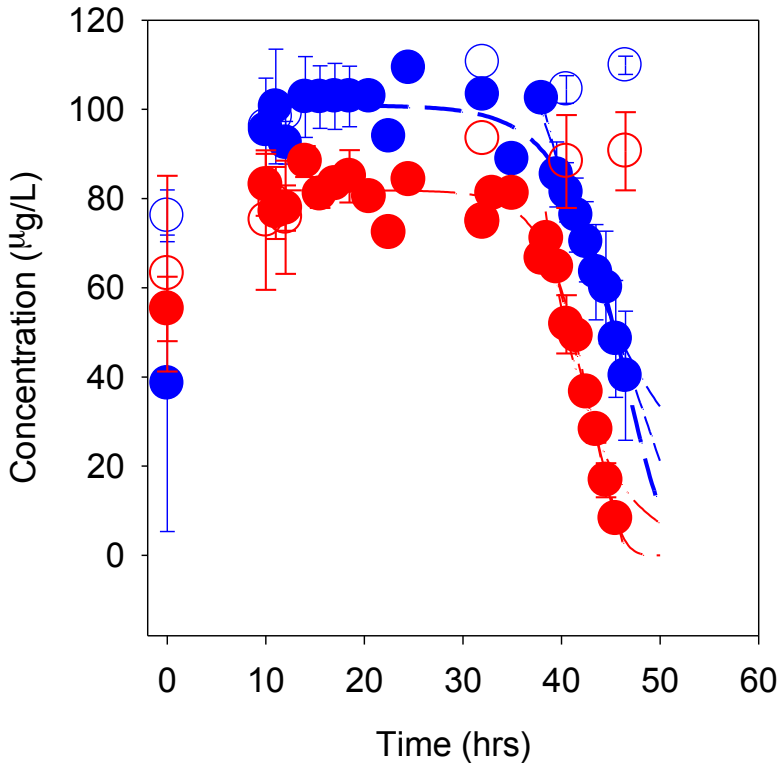

Figure S1. Degradation of genistein (red) and daidzein (blue) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent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

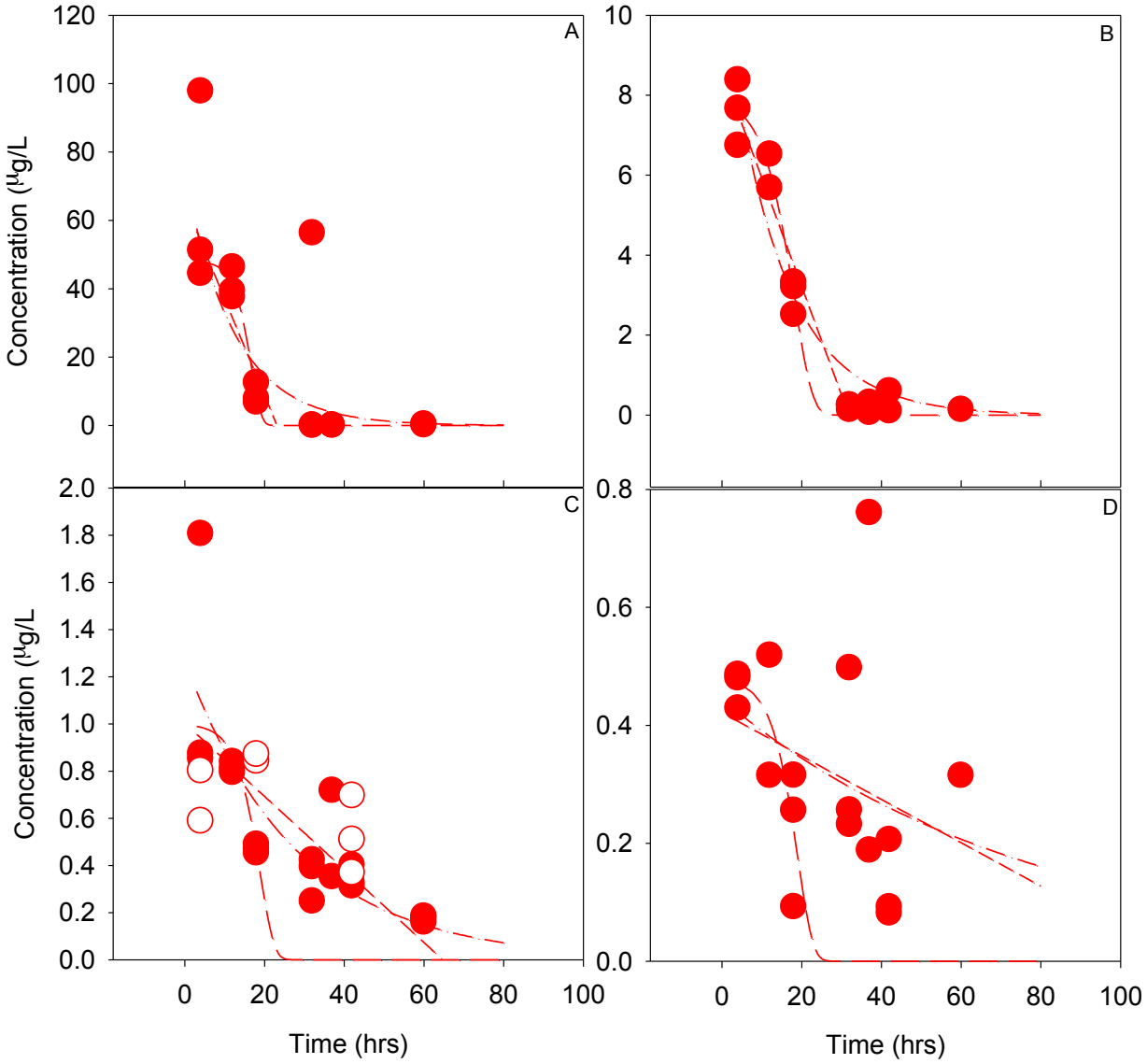

Figure S2. Degradation of genistein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and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

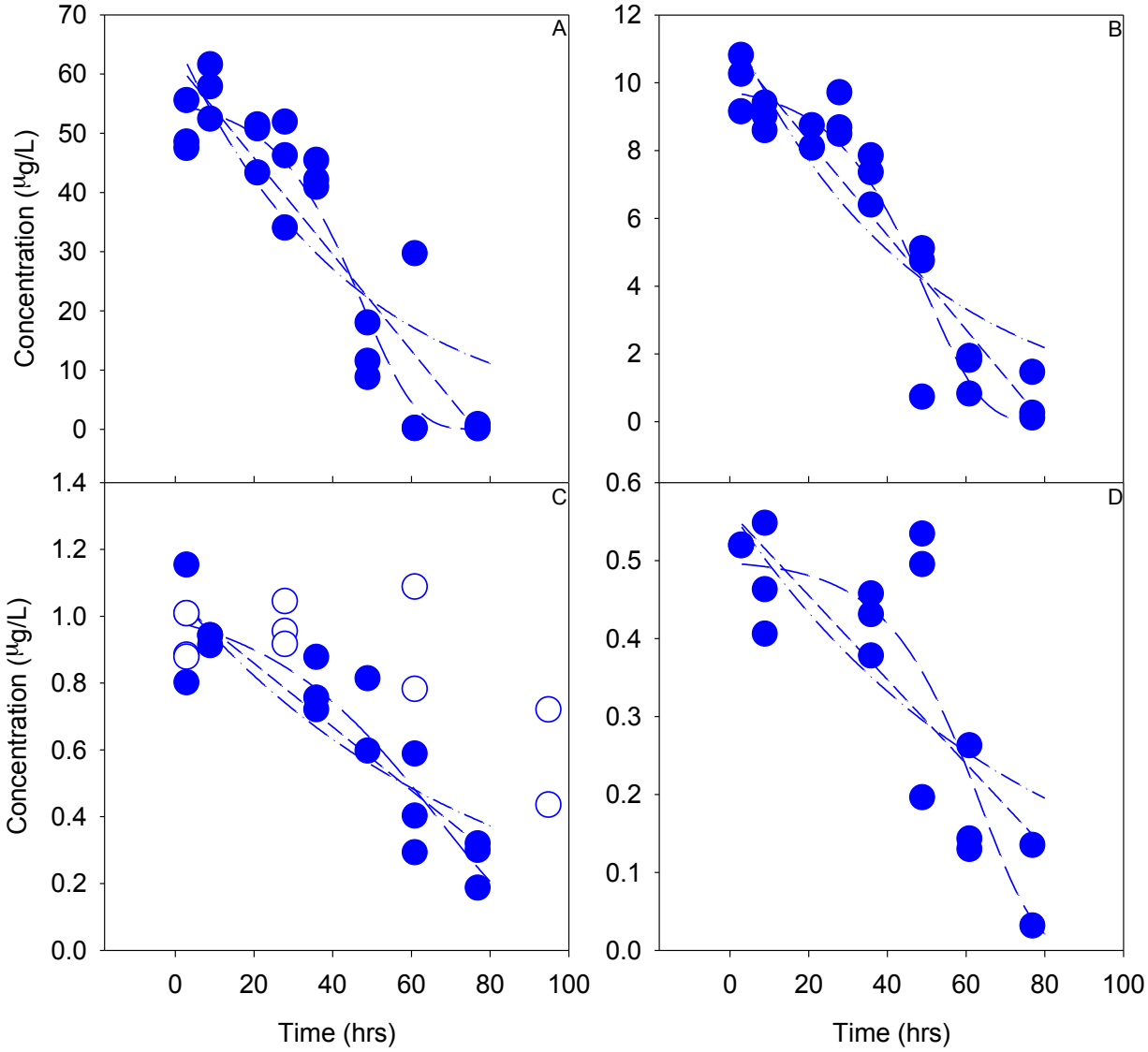

Figure S3. Degradation of daidzein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on June 26, 2013, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot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

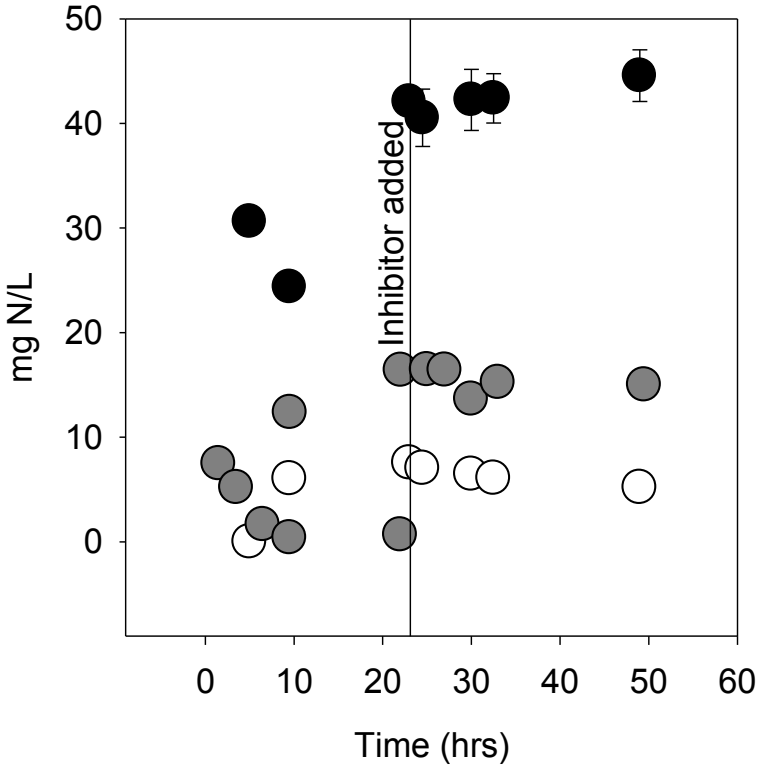

Figure S4. Concentrations of nitrite (black), nitrate (white), and ammonia (grey) in an enriched culture of nitrifying organisms before and after the addition of allylthiourea.

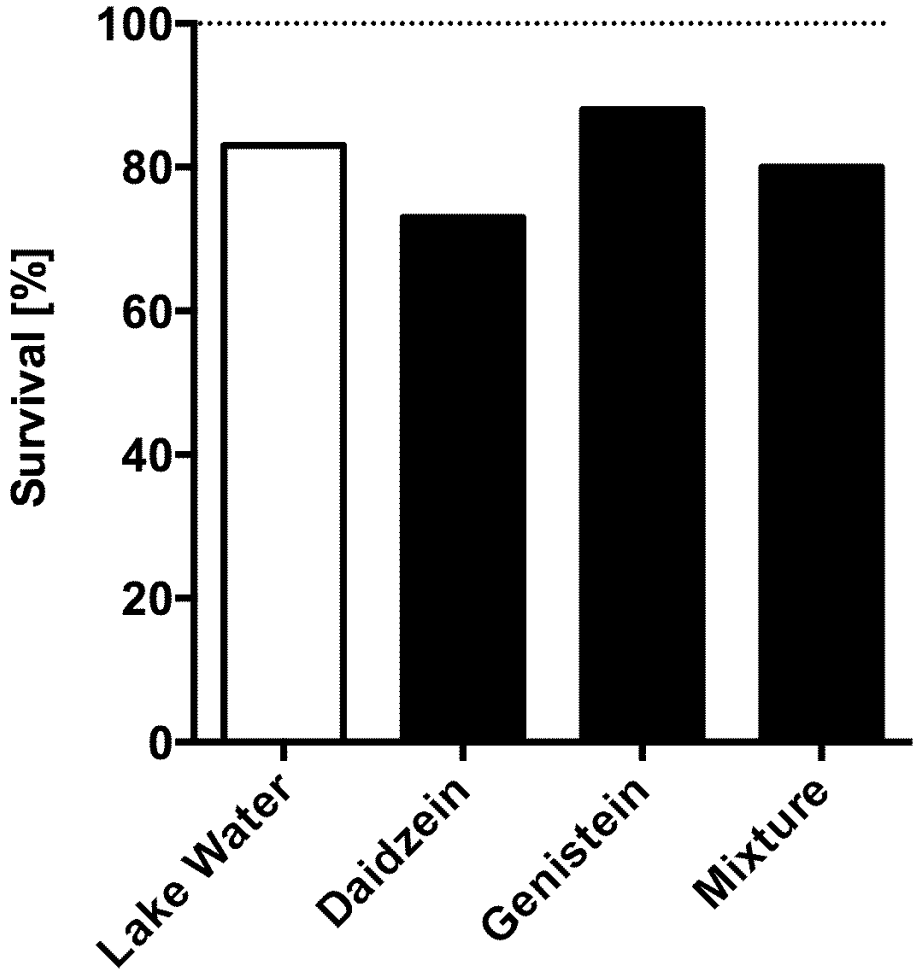

Figure S5. Percent larval fathead minnow survival during a 21-day exposure to the degradation compounds singly and in mixture. Initial cohorts were established using 75 larvae per treatment. Survival represents number of individuals remaining after behavioral testing.

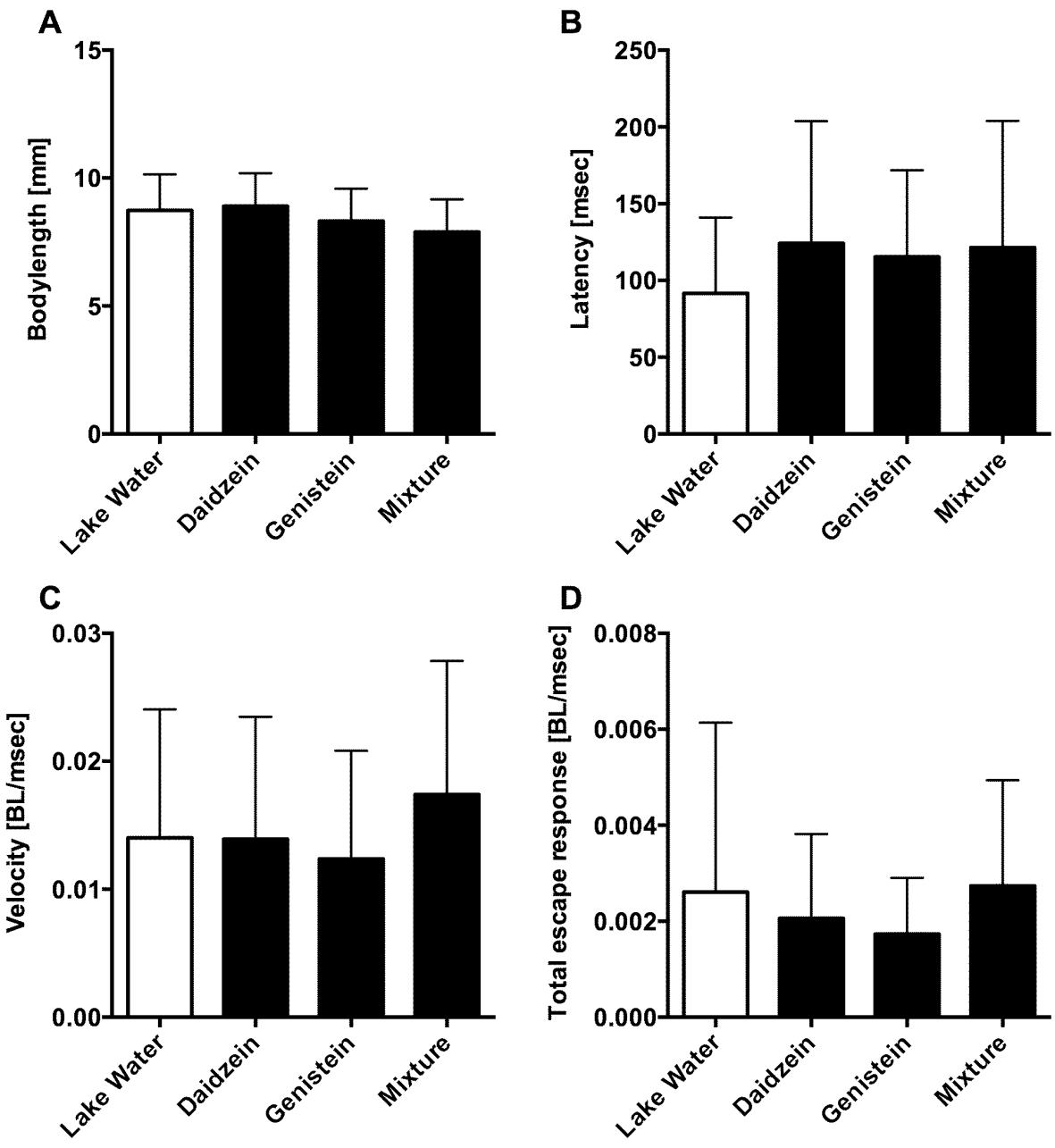

Figure S6. Larval fathead minnow escape performance following 21-day exposure to degraded phytoestrogen compounds. C-start escape performance was quantified using. (A) body length (mm); (B) mean latency (ms) from stimulus to response; (C) mean escape velocity relative to body length (BL/ms); (D) mean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

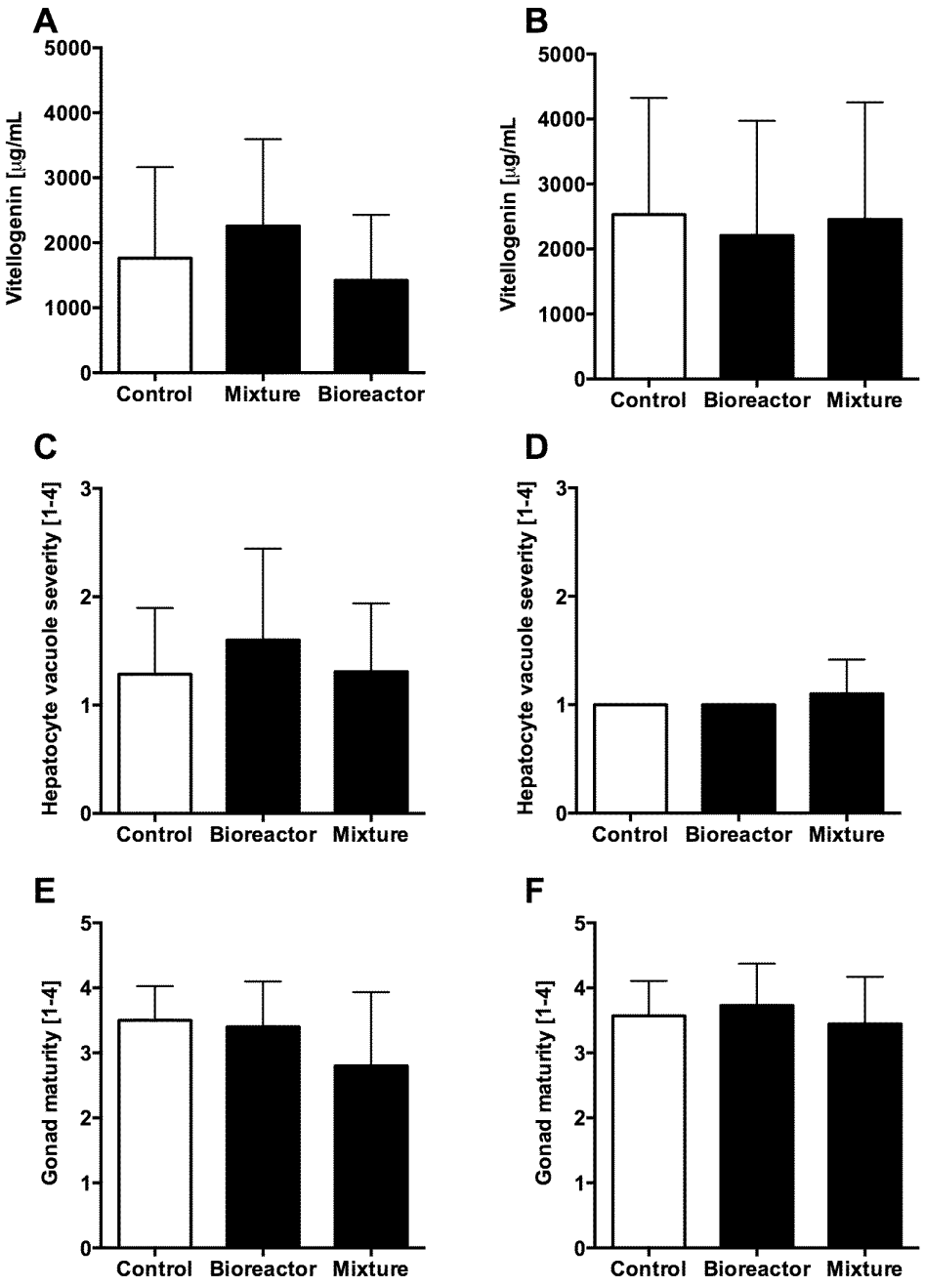

Figure S7. Mean ± standard error plasma vitellogenin concentrations (µg/mL) in male (A) and female (B) fathead minnows exposed for 21- days to the degraded phytoestrogen mixture (Daidzein, Genistein, and Formononetin 1,000 ng/L per compound). Severity of hepatocyte vacuole presence for male (C) and female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

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

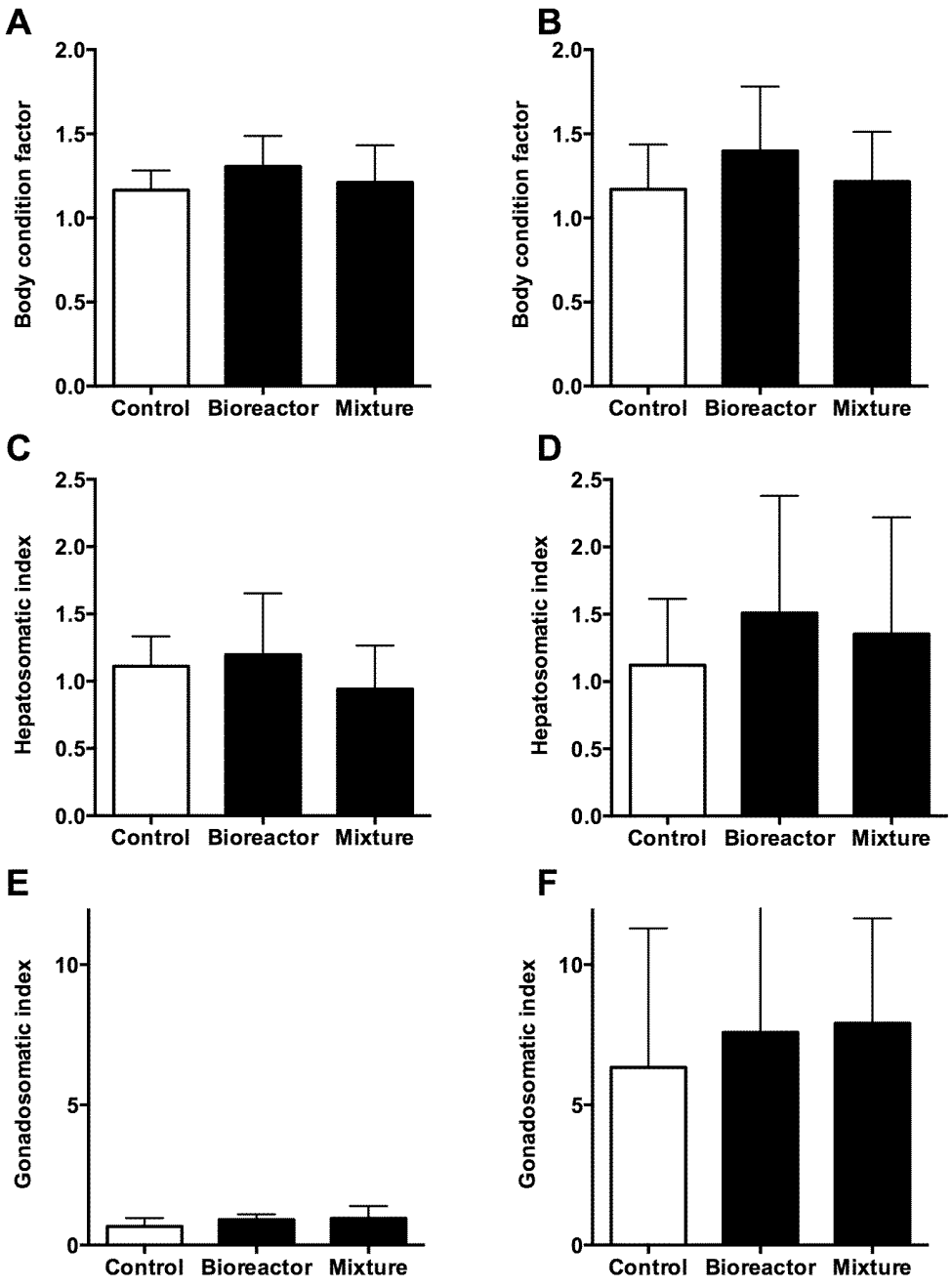

Figure S8. Body condition factor (A-male; B-female), hepatosomatic idnex (C,D) and gonadosomatic index (E,F) for fathead minnows exposed for 21 days to the degradation mixture.

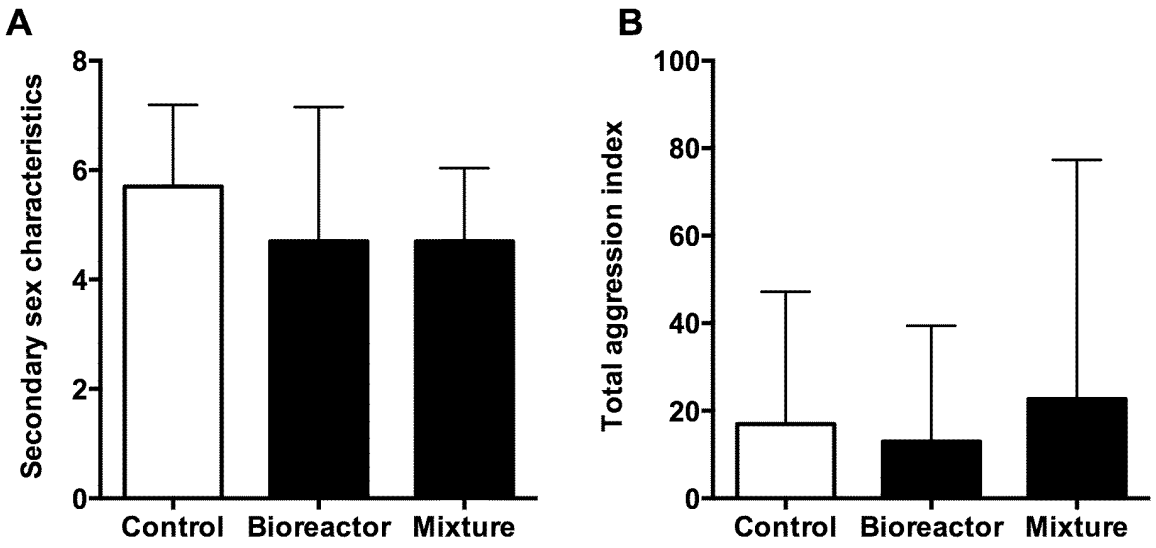

Figure S9. Mean ± standard error expression of secondary sex characteristics (A) and total aggression index (B) for male fathead minnows exposed 21-days to the degradation mixture.

REFERENCES

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