The Environmental Fate of the Phytoestrogens Genistein and Daidzein

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Abstract

Genistein and daidzein are two phytoestrogens, compounds that come from plants (especially legumes) and interact with the estrogen receptors present in humans, fish, and other animals. Although these compounds are naturally produced in the environment, they can become concentrated in the effluent of facilities treating the waste of plant-processing industries. They are also detected in run-off from fields where plants such as red clover are found. At environmentally-relevant concentrations, genistein and daidzein have been shown to produce reproductive, behavioral, and immunosuppressive effects in fish. This work develops the chemical and microbiological parameters necessary to predict the concentrations of genistein and daidzein in a body of water receiving a discharge containing the phytoestrogens. The attenuation processes studied include direct and indirect photolysis, sorption to settling particles, and biodegradation. Biodegradation is shown to be an extremely efficient removal process. Work by the Aquatic Toxicology Lab at Saint Cloud State shows that estrogenicity is removed by biodegradation, but some component of the biodegradation product mixture produces an androgenic or anti-estrogenic effect. More research is needed, therefore, to fully understand the exposure of aquatic wildlife to phytoestrogens and their degradates.

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Chapter 1: Introduction

Biofuels and Phytoestrogens

Phytoestrogens are compounds that come from plants and produce estrogenic effects in animals. Classes of phytoestrogens include isoflavones, mycotoxins, and coumestans. Coumestans have been detected in legumes, grasses, asters, and plants in the family *Chenopodiaceae*, which includes spinach and quinoa.[1] Mycotoxins are actually produced by fungi that infect plants. Isoflavones are produced by more than 60 different families of plants,[1] including high-production food crops such as rice, potatoes, tomatoes, onions, cucumbers, and grapes.[2–4] Of these, soy and kudzu show the highest concentrations of isoflavones.[5]

Soy is produced for both food and fuel and is produced in large quantities world-wide. Most soy grown for food is not eaten directly as soybeans, but processed into other forms before consumption, so processing of soy for both food and fuel may generate waste products that contain isoflavones. These waste products will become increasingly important as the production of soy biodiesel increases. United States biodiesel production has increased from 23 million gallons per year early in the 21st century to over 1 billion gallons in 2012.[6] To reach this level of production, over 4 billion gallons of soy oil were refined.[7] The current biodiesel production capacity is about 3 billion gallons per year, and the National Biodiesel Board has set a goal that 10% of the diesel market come from biodiesel by 2022.[6,8] According to the World Bank, U.S. diesel consumption in 2010 was approximately 124 billion kg, or 38 billion gallons, assuming a density of 0.85 kg/L.[9–11] The refining process of biodiesel is rather water-lean, using up to 3 gallons of water per gallon of biodiesel (with just 1 gallon/gallon consumed), compared to 7 gallons/gallon overall, and up to 4 gallons/gallon consumed for corn ethanol.[12]

Of the various phytoestrogens, isoflavones are the most important because of their high concentrations in soy, combined with their estrogenic activity. For example, although coumestrol, the main coumestan, has been shown to bind as strongly as genistein, an important isoflavone, to the estrogen receptor β and more strongly to the estrogen receptor α, it is found in soybeans at a concentration less than 1/10,000 that of genistein and daidzein.[2,13] In vivo, genistein has been shown to affect behavior, reproductive ability, and immune system function at concentrations of 1000 ng/L.[14-17] Other important isoflavones include daidzein and the less-strongly binding biochanin A and formononetin (Figure 1-1).[13]

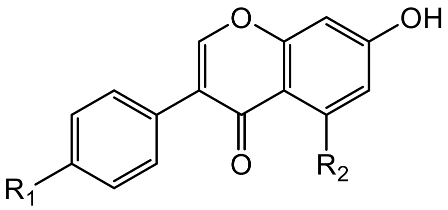


Figure 1-1. Structure of isoflavones (genistein: $R_1 = R_2 =$ OH, daidzein: $R_1 =$ OH, $R_2 =$ H, biochanin A: $R_1 = OCH_3$, $R_2 =$ OH, and formononetin: $R_1 = OCH_3$, $R_2 =$ H).

Levels in the Environment

Because they are found at high concentrations in soy as well as in other plants, isoflavones are found in a wide variety of effluents from plant-based industries[18–20] and municipal wastewater treatment plants, which may receive waste from plant-based industries in addition to human excretions containing isoflavones.[19–25] The first detection of phytoestrogens in the environment dates to the 1950s, when sheep grazing on red clover began having reproductive troubles which were traced to the estrogenic activity of genistein in the clover.[26] Since then, phytoestrogens have been detected around the world at various concentrations and under various conditions.

Recently, the first detections of phytoestrogens in soil and manure were completed in Switzerland.[27] Less than 100 ng/g of genistein and daidzein, but up to 3.35 μg/g of formononetin and 0.258 μg/g of biochanin A were found in the soil samples. In cow manure, up to 6.18, 4.31, 2.37, and 0.809 μg/g of formononetin, daidzein, biochanin A, and genistein were detected. Equol, a metabolite of daidzein and formononetin produced in mammalian digestive tracts, was detected at concentrations up to 387 μg/g. The soils investigated were dominated by red clover, which contains formononetin and biochanin A at a much higher concentration than genistein and daidzein.[28] In the same area, river water samples were found to contain up to 22 ng/L of formononetin or 12 ng/L of biochanin A, but genistein and daidzein were not detected. The source to the river water is likely drainage water from the fields of clover, which was found to contain 157, 22, 14, and 30 ng/L of formononetin, biochanin A, genistein, and daidzein, respectively.[29] Additionally, over a three year period, concentrations of formononetin, genistein, and daidzein concentrations were highest after a rainfall event preceded by cut grass being dried on the field, suggesting phytoestrogens are subject to first-flush effects. The same study concluded that on average, of the isoflavones produced by plants or applied to soil with manure, only 0.11% were emitted in drainage water, strongly suggesting a role for sorption to soils and biodegradation.[30]

In Rio de Janeiro, Brazil, genistein and daidzein were detected in a canal in one of two sampling campaigns at concentrations up to 366 and 276 ng/L, respectively.[31] Daidzein was also detected at 35 ng/L in a lagoon in Rio de Janeiro during the other sampling campaign. Genistein was also detected in a smaller city upstream of Rio de Janeiro during both sampling campaigns, but at concentrations of <15 ng/L.

In Portugal, the Douro River estuary was sampled quarterly for genistein, daidzein, and biochanin A at high and low tide. Biochanin A was detected during all four seasons, at concentrations up to 191.4 ng/L, but genistein and daidzein were only detected in the summer. In contrast, biochanin A was at its lowest levels during the summer. At its highest levels during the spring, biochanin A was detected at all nine study sites at both high and low tide. The authors were not able to decipher a spatial gradient for any of the compounds during any season, which they attribute to the "artificial hydrodynamics" of the system caused by a dam upstream of the sampling area.[32]

The same group also studied the Mondego River estuary with quite different results.[33] Daidzein was not detected in summer or fall, but it was detected during the winter at all eight sites during high tide and at five sites during low tide. In the spring, it was detected inconsistently at concentrations up to 526 ng/L. Genistein was detected in only three samples over the entire study. All three samples occurred during the spring sampling. A high tide sample taken at the site closest to the Atlantic Ocean had 22.5 ng/L, while a low tide sample taken at the next closest site in the north arm of the estuary had 32.6 ng/L. The third sample was taken from the furthest upstream site at low tide and had 507 ng/L genistein. Biochanin A was not nearly as prevalent in the Mondego River estuary, detected at only a handful of sites in fall and spring, none in summer, and at lower concentrations in the winter compared to the Douro River estuary (up to 29.3 ng/L). The two sites with the highest total concentrations of all three measured phytoestrogens were in the most heavily agricultural sites; one directly downstream of an agriculturally dominated tributary, and the other flowing between two agricultural fields. The authors attribute the higher concentrations in spring (compared to winter) in the Mondego River estuary to the planting season.[33] The authors do not offer an explanation for the lower detection frequency of biochanin A in spring, summer, and fall.

In agricultural streams in Iowa, phytoestrogens were detected at several locations and timepoints. The highest concentrations for genistein, daidzein, and formononetin (up to 8 ng/L, 41 ng/L, and 12.3 ng/L respectively) were detected after spring snowmelt, providing addition support for the first-flush effects observed in Swiss pastures.[27,30] Biochanin A was detected below the limit of quantification in the spring snowmelt sample, but its highest concentration (5.6 ng/L) was found in July, in contrast to the Portuguese studies.

In Italy, phytoestrogens were detected at low concentrations (<10 ng/L) in the Tiber River in Rome but were not detected in a nearby volcanic lake, which receives only rainwater and groundwater as inputs.[23,24]

In Japan, exceptionally high concentrations of genistein and daidzein (143.4 μg/L and 42.9 μg/L, respectively) were detected in the Kanzaki River near Osaka.[34] Although there are food and wood pulp processing facilities in the area, no single point source was identified. The authors detected no phytoestrogens using the YES assay when sampling 2 km downstream of the site where phytoestrogens were first detected, suggesting that dilution and other in-stream processes are mitigating the estrogenic effects of genistein and daidzein.

Potential Fate Processes

Fate processes suggested by authors of studies detecting phytoestrogens in the environment include sorption to soils, biodegradation in soils, and dilution.[30,34] Successful removal of a large proportion of phytoestrogens in activated sludge and trickling filter wastewater treatment plants has been observed, suggesting that biodegradation or sorption to sewage sludge or other solids are potential avenues for phytoestrogen removal from natural waters.[19,20,24] Though not specifically mentioned by previous authors, it seems likely that photolysis will also play a role in phytoestrogen fate in the environment, given phytoestrogens' strong ultraviolet light absorbance and structural similarity to other environmental contaminants that have been shown to undergo photolysis in the environment.[35–39]

Photolysis. Photolysis occurs in the environment when a molecule absorbs a photon, ultimately leading to a change in the chemical structure of the compound of interest. Direct photolysis refers to a compound of interest (in this case, a phytoestrogen) absorbing the photon and reacting, while indirect photolysis refers to the reaction of the compound of interest with a reactive species generated by another molecule's absorption of a photon (Figure 1-2). Reactive species may include triplet-state natural organic matter, singlet oxygen, hydroxyl radical, and other reactive oxygen species.

Direct photolysis kinetics are described by the equation

$$\frac{dC}{dt} = -\phi k_a[C] \tag{1-1}$$

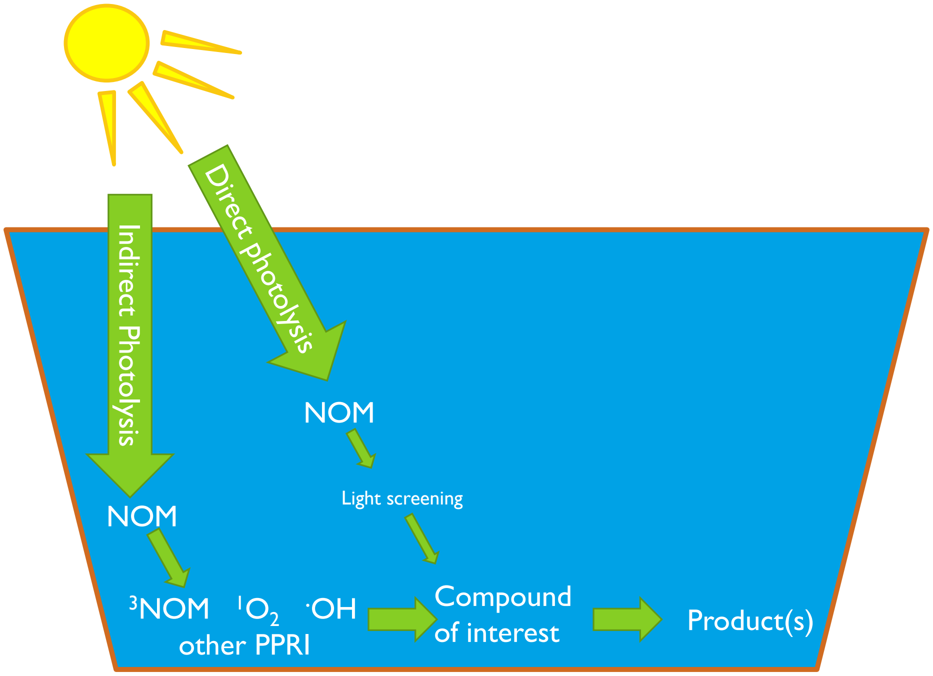


Figure 1-2. Photolytic pathways for the degradation of a compound of interest. Indirect photolysis occurs when natural organic matter (NOM) absorbs a photon and generates a photochemically produced reactive intermediate (PPRI) such as triplet natural organic matter (^{3}NOM), singlet oxygen ($^1O_2$), or hydroxyl radical ($^{\cdot}$OH).

where k_a is the rate at which the compound absorbs light, ϕ is the quantum yield, and $[C]$ is the concentration of the compound of interest. The light absorption rate k_a is determined by the overlap of the solar spectrum and the absorption spectrum of the molecule. The quantum yield is the fraction of molecules that go on to react after absorbing a photon. Knowing the quantum yield and molar absorptivity of a compound along with the irradiation spectrum thus enables the prediction of the kinetics of the direct photolysis of C.

Indirect photolysis occurs according to the equation

$$\frac{dC}{dt} = -k_{PPRI}[C][PPRI] \tag{1-2}$$

where k_{PPRI} is the 2nd-order rate constant for the reaction between C and the photochemically produced reactive intermediate (PPRI). In the environment, PPRIs tend to be found at very low (10^{-12} to 10^{-18} M), but steady-state concentrations, so knowing the 2nd-order rate constant and the steady-state concentration of a PPRI enables prediction of the indirect photolysis kinetics for C.

Sorption. Sorption refers to a compound of interest associating with a particle or colloid in the environment either by forming chemical bonds (chemisorption) or by weak associations such as van der Waals forces (physisorption). A molecule on the outer surface of a particle is adsorbed, while a molecule that becomes associated with the inside of a particle is absorbed. Adsorption occurs by a number of mechanisms, classified by Mackay and Vasudevan as Type I and Type II interactions.[39] Type I interactions occur to some extent for all organic molecules and include hydrophobic partitioning and electron donor-acceptor interactions. Type II interactions occur only for ionogenic molecules, such as isoflavones, and include cation exchange, anion exchange, cation bridging, and surface complexation.[39] Isoflavones contain no functional groups that take on a positive charge, so cation exchange is not a likely sorption mechanism. Due to the phenolic content of isoflavones, the other three mechanisms may play a role. Because anion exchange is typically a minor process for most soils and sediments, cation bridging and surface complexation are the most likely Type II interactions for phytoestrogens.[40] Cation bridging requires that a particle contain a negative surface site with a sorbed cation, and surface complexation requires that a particle contain surface Fe or Al atoms.

A comprehensive description of the distribution coefficient, K_D (L/kg), is shown in equation 1-3:

$$K_D = \frac{C_{sorbed}}{C_{dissolved}} = K_{oc} f_{oc} + K_{min} A_{surf} + K_{rxn} \sigma_{surf\ rxn} A_{surf} \tag{1-3}$$

where K_{oc} (L/kg) is the partitioning coefficient for organic carbon, f_{oc} (-) is the fraction of organic carbon in the environmental solid, K_{min} (L/m^2) is the distribution coefficient for a compound attracted to a mineral surface due to hydrophobic partitioning or weak electrostatic interactions, A_{surf} is the specific surface area of the solid (m^2/kg), K_{rxn} is the (L/mol) distribution coefficient for the compound sorbing to a surface site via chemical reaction (such as surface complexation), and $\sigma_{surf\ rxn}$ (mol/m^2) is the concentration of appropriate sites in the solid.[41] Knowing which parameters are most likely to be contributing to the sorption of a compound, along with the relevant experimental values, will enable the prediction of attenuation of that compound due to sorption.

Biodegradation. To date, few if any efforts have been made to observe the biological removal of phytoestrogens in natural waters. Removal of phytoestrogens to varying extents in biologically active wastewater treatment has been observed, suggesting that biodegradation could also act as an in-stream removal process.[20,24] It is unclear, however, which organisms are responsible for phytoestrogen degradation in treatment plants and by what mechanisms the organisms degrade the phytoestrogens. One possibility is that phytoestrogens are cometabolically degraded, as has been observed for steroid estrogens, bisphenol A, and nonylphenol, and other phenolic, wastewater-derived contaminants.[42–45] Several of the authors demonstrating cometabolism of steroid and xenoestrogens also demonstrated the estrogen degrading capability of heterotrophic organisms in activated sludge systems.[43–45] Because steroid estrogens, xenoestrogens, and phytoestrogens are typically not found in the environment at concentrations above the μg/L range, microorganisms able to use these molecules for growth would likely need to be multiple substrate utilizers.

Motivation and Outline of Thesis

The variety of concentrations of phytoestrogens detected in the environment around the world suggests various sources of phytoestrogens and site-dependent removal mechanisms. To understand phytoestrogens' exposure to aquatic organisms in waste-impacted natural waters, it is necessary to know the chemical properties (including pK_a, ϕ, k_{PPRI}, K_D, and biodegradability) of phytoestrogens, along with the site-specific parameters that affect phytoestrogen fate (pH, solar exposure, suspended sediment concentration, sediment composition, presence of phytoestrogen degrading microorganisms). This study aims to quantify the relevant chemical properties so that removal at a given site can be calculated if the site-specific parameters are known. If environmental processes do not remove phytoestrogens efficiently enough to prevent harm to aquatic wildlife, alternate waste treatment decisions can be made.

Chapter 2 describes the photolytic fate of genistein and daidzein. The pK_a values are determined, along with the UV-visible spectra of each molecule at a variety of pH values. Direct photolysis is quantified, the mechanism and importance of indirect photolysis is investigated, and 2nd-order rate constants for hydroxyl radical and singlet oxygen are determined. The rate of direct photolysis, pK_a, and UV-visible spectra are used to calculate the quantum yield of each compound in total at several pH values, and the quantum yield of each protonation state of each compound, so that the rate of direct photolysis can be estimated under varying pH and solar exposure conditions. In Chapter 3, sorption to minerals and river sediment is quantified. Distribution coefficients for goethite, kaolinite, calcium-exchanged montmorillonite, and sodium-exchanged montmorilllonite are determined at several pH values and ionic strengths, using sorption isotherms. The mechanism of sorption is also probed using sorption edges at several ionic strength values. In Chapter 4, the biodegradation of genistein and daidzein is investigated, using batch reactors at several initial concentrations. In Chapter 5, the environmental fate of genistein and daidzein is modeled under different conditions, demonstrating that the relative importance of each attenuation process can vary by site. This type of modeling can be used to assess the risk posed to aquatic wildlife living in the vicinity of phytoestrogen discharges.

References

(1) Lapcík, O. Isoflavonoids in non-leguminous taxa: a rarity or a rule? *Phytochemistry 68*, 2909–16.

(2) Thompson, L. U.; Boucher, B. A.; Liu, Z.; Cotterchio, M.; Kreiger, N. Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. *Nutr. Cancer* **2006**, *54*, 184–201.

(3) Liggins, J.; Bluck, L. J. C.; Runswick, S.; Atkinson, C.; Coward, W. A.; Bingham, S. A. Daidzein and genistein contents of vegetables. *Brit. J. Nutr.* **2000**, *84*, 717–725.

(4) De Sanctis, F.; Silvestrini, M. G.; Luneia, R.; Botondi, R.; Bellincontro, A.; Mencarelli, F. Postharvest dehydration of wine white grapes to increase genistein, daidzein and the main carotenoids. *Food Chem.* **2012**, *135*, 1619–25.

(5) Mazur, W.; Adlercreutz, H. Naturally occurring oestrogens in food. *Pure Appl. Chem.* **1998**, *70*, 1759–1776.

(6) National Biodiesel Board. Biodiesel Basics http://www.biodiesel.org/what-is-biodiesel/biodiesel-basics. Accessed 7-22-2013.

(7) Energy Information Administration U.S. Inputs to Biodiesel Production http://www.eia.gov/biofuels/biodiesel/production/table3.pdf. Accessed 7-22-2013.

(8) Biodiesel Industry Aims For 10% of On-Road Diesel Market by 2022 http://www.biofuelsjournal.com/articles/Biodiesel_Industry_Aims_For_10__of_On_Road_Diesel_Market_by_2022-130159.html. Accessed 7-22-2013.

(9) Chevron Corporation. Diesel Fuels Technical Review **2007**, 116 pp.

(10) International Road Federation, World Road Statistics, and I. E. A. Road sector diesel fuel consumption per capita http://data.worldbank.org/indicator/IS.ROD.DESL.PC. Accessed 7-22-2013.

(11) United Nations Population Division, United Nations Statistical Division, Eurostat: Demographic Statistics, and Secretariat of the Pacific Community, and U. S. C. B. I. D. Population, total http://data.worldbank.org/indicator/SP.POP.TOTL. Accessed 7-22-2013

(12) Committee on Water Implications of Biofuels Production in the United States, N. R. C. *Water Implications of Biofuels Production in the United States*; National Academies Press: Washington, D.C., 2008; p. 88.

(13) Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B.; Gustafsson, J. a Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **1998**, *139*, 4252–63.

(14) 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.

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

(16) 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 Persp.* **2003**, *111*, 1158-1163.

(17) Rearick, D. C.; Fleischhacker, N. T.; Kelly, M. M.; Arnold, W. A.; Novak, P. J.; Schoenfuss, H. L. Environmenal occurrence of phytoestrogens and exposure effects on fathead minnows. In preparation.

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

(19) Ferrer, I.; Barber, L. B.; Thurman, E. M. Gas chromatographic-mass spectrometric fragmentation study of phytoestrogens as their trimethylsilyl derivatives: identification in soy milk and wastewater samples. *J. Chromatogr. A* **2009**, *1216*, 6024–32.

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

(21) Spengler, P.; Körner, W.; Metzger, J. W. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. *Environ. Toxicol. Chem.* **2001**, *20*, 2133–2141.

(22) Pawlowski, S.; Ternes, T.; Bonerz, M.; Kluczka, T.; van der Burg, B.; Nau, H.; Erdinger, L.; Braunbeck, T. Combined in situ and in vitro assessment of the estrogenic activity of sewage and surface water samples. *Toxicol. Sci.* **2003**, *75*, 57–65.

(23) 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.

(24) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P.; Samperi, R.; Laganà, A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**, *531*, 229–237.

(25) 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 Sp.* **2006**, *20*, 2411–2418.

(26) Biggers, J. D.; Curnow, D. H. Oestrogenic activity of subterranean clover. I. The oestregenic activity of genistein. *Biochem. J.* **1954**, *58*, 278–282.

(27) 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.

(28) Sivesind, E.; Seguin, P. Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *J. Agr. Food Chem.* **2005**, *53*, 6397–402.

(29) 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. Agr. Food Chem.* **2007**, *55*, 8339–8345.

(30) Hoerger, C. C.; Wettstein, F. E.; Bachmann, H. J.; Hungerbühler, K.; Bucheli, T. D. Occurrence and mass balance of isoflavones on an experimental grassland field. *Environ. Sci. Technol.* **2011**, *45*, 6752–60.

(31) Kuster, M.; Azevedo, D. A.; López de Alda, M. J.; Aquino Neto, F. R.; Barceló, D. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). *Environ. Int.* **2009**, *35*, 997–1003.

(32) Ribeiro, C.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. *Arch. Environ. Con. Tox.* **2009**, *56*, 1–11.

(33) Ribeiro, C.; Pardal, M. A.; Martinho, F.; Margalho, R.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Distribution of endocrine disruptors in the Mondego River estuary, Portugal. *Environ. Monit. Assess.* **2009**, *149*, 183–93.

(34) 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.

(35) 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.

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

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

(38) 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.

(39) MacKay, A. A.; Vasudevan, D. Polyfunctional ionogenic compound sorption: challenges and new approaches to advance predictive models. *Environ. Sci. Technol.* **2012**, *46*, 9209–23.

(40) Sposito, G. *The Chemistry of Soils*; Oxford University Press, USA, 2008; p. 329.

(41) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

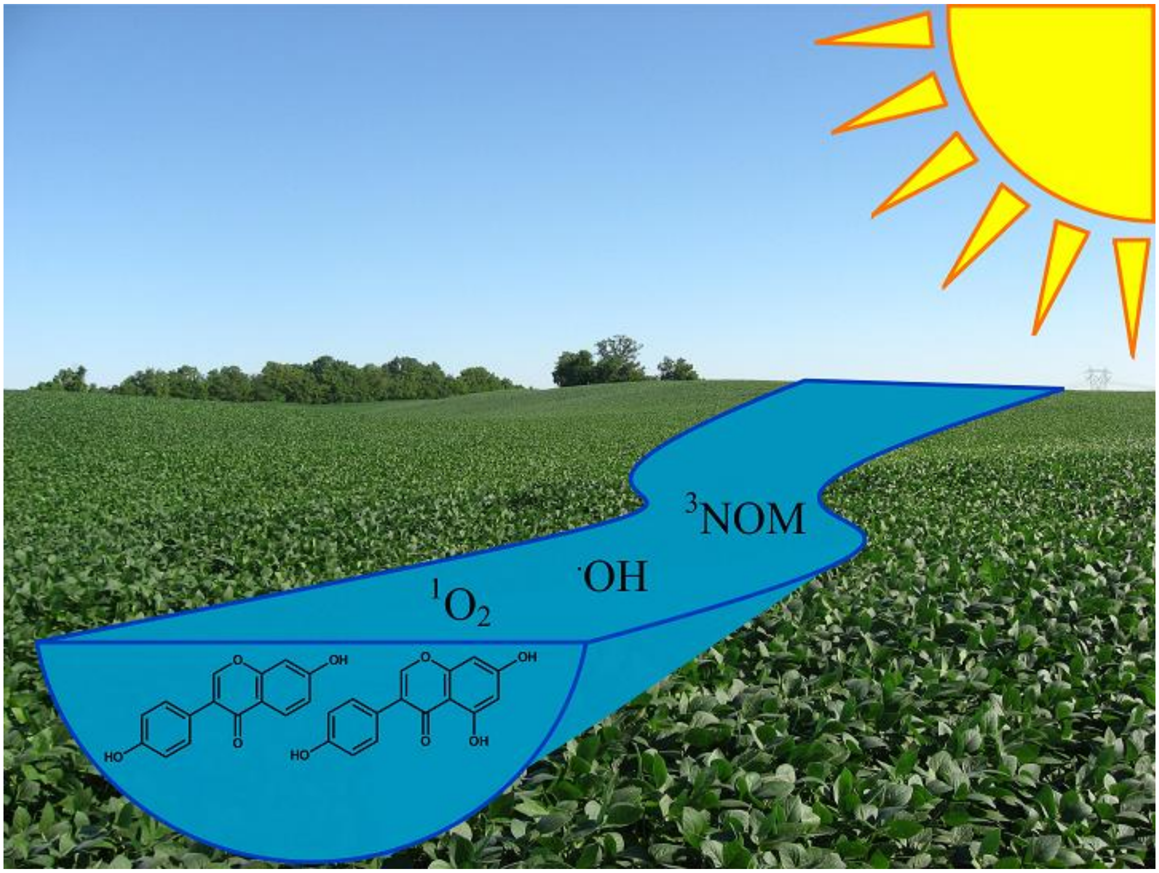
(42) Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Res.* **2004**, *38*, 2323–2330.

(43) Vader, J.; Van Ginkel, C.; Sperling, F.; De Jong, J.; De Boer, W.; De Graaf, J.; Van Der Most, M.; Stokman, P. Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere* **2000**, *41*, 1239–1243.

(44) Roh, H.; Subramanya, N.; Zhao, F.; Yu, C.-P.; Sandt, J.; Chu, K.-H. Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria. *Chemosphere* **2009**, *77*, 1084–9.

(45) Kim, J. Y.; Ryu, K.; Kim, E. J.; Choe, W. S.; Cha, G. C.; Yoo, I.-K. Degradation of bisphenol A and nonylphenol by nitrifying activated sludge. *Process Biochem.* **2007**, *42*, 1470–1474.

Chapter 2: Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein



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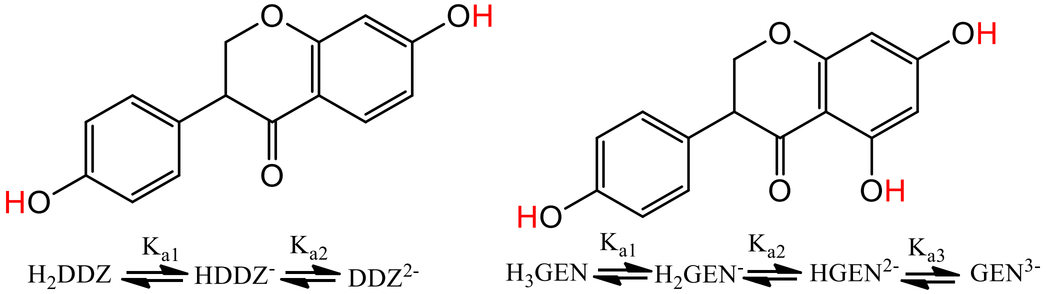
Introduction

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 two to three 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.

Scheme 1



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 ($^{\cdot}OH$) 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 enhanced by $^{\cdot}OH$ 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 $^{\cdot}OH$, singlet oxygen ($^{1}O_2$), or ^{3}NOM.[22–24]

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

$$\frac{dC}{dt} = -\phi k_a [C] \tag{2-1}$$

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 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

$$\Phi_{dc} = \frac{k_{dc}}{k_{da}} \sum_{\lambda} \frac{\varepsilon_{\lambda a} L_{\lambda} \lambda_{range}}{\varepsilon_{\lambda c} L_{\lambda} \lambda_{range}} \Phi_{da} \tag{2-2}$$

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 $^1O_2$, $^{\cdot}OH$, ^3NOM, 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 Appendix A

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 minutes 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, $k_{^1O_2}$ =1.2×10^8 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 exposed in quartz tubes to simulated sunlight solar simulator for 2 hours, with sub-samples removed every 20 minutes.

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}=5.9×10^9 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 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 2-1. The spectra were used to determine the pK_a values of genistein and daidzein by fitting spectral data to the following equations:

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

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

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

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. Equations 2-4 and 2-5 are expanded forms of equation 2-3, 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 equations 2-4 (for genistein) and 2-5 (for daidzein) to the UV-Vis spectra of the respective phytoestrogens at various pH values shown in Figure 2-1, 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 2-1, 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.

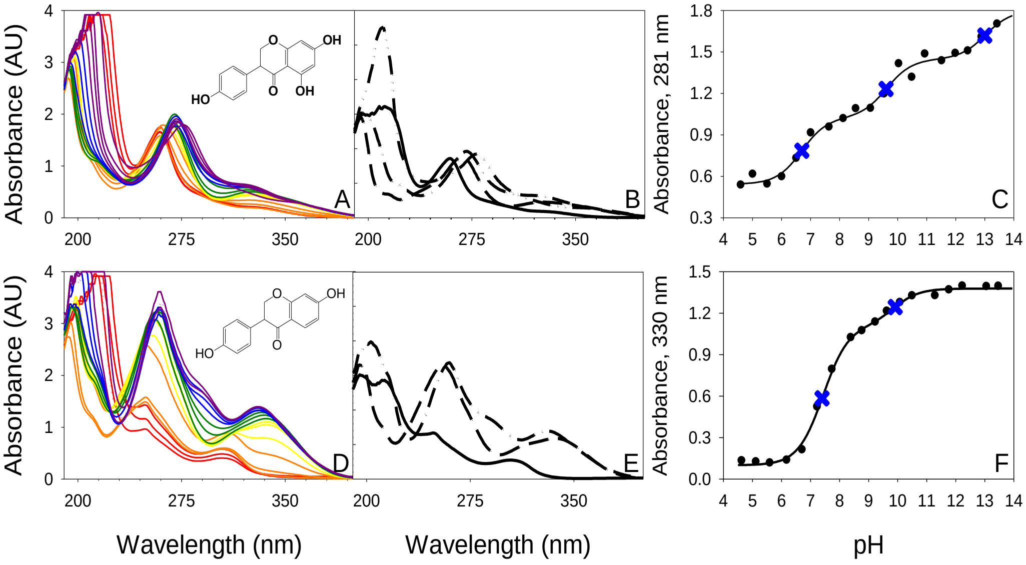


Figure 2-1. UV-Visible spectra collected as a function of pH (panel A for genistein and D for daidzein, pH increases from red to purple) used to determine pK_a values. The pK_a values determined were 6.70 ± 0.39, 9.62 ± 0.45, and 13.0 ± 1.1 for genistein, and 7.43 ± 0.10 and 9.88 ± 0.35 for daidzein (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 Appendix A.

Determination of Quantum Yields

The quantum yield of photolysis was determined at each pH for each compound using equation 2-2. The first term in equation 2-2, $\left(\frac{k_{dc}}{k_{da}}\right)$, was calculated by plotting the concentration of substrate ($[C]/[C_0]$) versus the concentration of actinometer ($[\text{actinometer}]/[\text{actinometer}]_0$) at each point on a log-log scale, and taking the slope of the resulting plot as $\left(\frac{k_{dc}}{k_{da}}\right)$. These plots, generated using data from the solar exposure experiments, are presented in Figure A-1 of Appendix A. Spectra of genistein and daidzein at each experimental pH were calculated using the component spectra (Figure 2-1, panels B and E, determined using the method of Boreen et al.[24,32] as described in Appendix A). 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 2-1. 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 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.

Table 2-1 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

<table>
<tr><th colspan="5">Observed</th></tr>
<tr><th></th><th>pH</th><th>Φ (-)</th><th>k_{OH} (M^{-1} s^{-1})</th><th>$k_{^1O_2}$ (M^{-1} s^{-1})</th></tr>
<tr><td>Genistein</td><td>5</td><td>1.3×10^{-4}</td><td>8.73 ± 0.31[a,b] × 10^9</td><td>3.57 ± 0.08 ×10^7 [c]</td></tr>
<tr><td></td><td>8.7</td><td>9.4×10^{-6}</td><td></td><td></td></tr>
<tr><td></td><td>11</td><td>7.2×10^{-5}</td><td></td><td></td></tr>
<tr><td></td><td>12</td><td>2.9×10^{-4}</td><td></td><td></td></tr>
<tr><td>Daidzein</td><td>5</td><td>4.7×10^{-4}</td><td>6.94 ± 0.09[b] × 10^9</td><td>1.84 ± 0.03 × 10^7 [c]</td></tr>
<tr><td></td><td>8.7</td><td>6.6×10^{-4}</td><td></td><td></td></tr>
<tr><td></td><td>12</td><td>1.4×10^{-4}</td><td></td><td></td></tr>
<tr><th colspan="5">Calculated</th></tr>
<tr><td colspan="2">protonation state</td><td>Φ (-)</td><td>protonation state</td><td>Φ (-)</td></tr>
<tr><td colspan="2">H_3GEN</td><td>6.9×10^{-5}</td><td>H_2DDZ</td><td>3.9×10^{-4}</td></tr>
<tr><td colspan="2">H_2GEN^-</td><td>3.3×10^{-6}</td><td>$HDDZ^-$</td><td>7.5×10^{-4}</td></tr>
<tr><td colspan="2">$HGEN^{2-}$</td><td>5.0×10^{-5}</td><td>DDZ^{2-}</td><td>3.1×10^{-5}</td></tr>
<tr><td colspan="2">GEN^{3-}</td><td>3.2×10^{-3}</td><td></td><td></td></tr>
</table>

[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.

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:

$$k_{dc,total} = k_{dc,\chi_1} + k_{dc,\chi_2} + k_{dc,\chi_3} + k_{dc,\chi_4} \qquad (2\text{-}6)$$

These values can all be divided by k_{da}, which yields a relative rate value $\frac{k_{dc,\chi_i}}{k_{da}}$that can be used to calculate the quantum yield of each protonation state. Equation 2-7 was used to determine $\frac{k_{dc,\chi_i}}{k_{da}}$,

$$\begin{bmatrix} \chi_{1,pH\ 5} & \cdots & \chi_{4,pH\ 5} \\ \vdots & \ddots & \vdots \\ \chi_{1,pH\ 12} & \cdots & \chi_{4,pH\ 12} \end{bmatrix} \begin{bmatrix} \frac{k_{dc,\chi_1}}{k_{da}} \\ \vdots \\ \frac{k_{dc,\chi_4}}{k_{da}} \end{bmatrix} = \begin{bmatrix} \frac{k_{dc,total,pH\ 5}}{k_{da}} \\ \vdots \\ \frac{k_{dc,total,pH\ 12}}{k_{da}} \end{bmatrix} \tag{2-7}$$

where $\frac{k_{dc,\chi_i}}{k_{da}}$ is the rate of transformation of protonation state *i* relative to the transformation of the actinometer used to measure $\frac{k_{dc,total}}{k_{da}}$, 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 $\frac{k_{dc,total}}{k_{da}}$ 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 $\left(\frac{k_{dc}}{k_{da}}\right)$ 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

$$k_{da} = \phi_{da} \sum_{\lambda} \varepsilon_{\lambda} L_{\lambda} \tag{2-8}$$

where $\sum_{\lambda} \varepsilon_{\lambda} L_{\lambda}$ 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 $\left(\frac{k_{dc}}{k_{da}}\right)$ for genistein at pH 5 from a PNAP actinometer basis to a PNA actinometer basis. The $\left(\frac{k_{dc}}{k_{da}}\right)$ 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 2-1. Using the component spectra and the component quantum yields, the fraction of direct photolysis attributable to each protonation state can be determined, according to equation 2-1.

As shown in Table 2-1, 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 2-2A). 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.

The singly deprotonated species of daidzein has the highest quantum yield, and dominates direct photolysis rates at pH 7 and above (Figure 2-2B). 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 2-2 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 equation 6 and using the solar spectrum for summer 40°N from ref. 25.

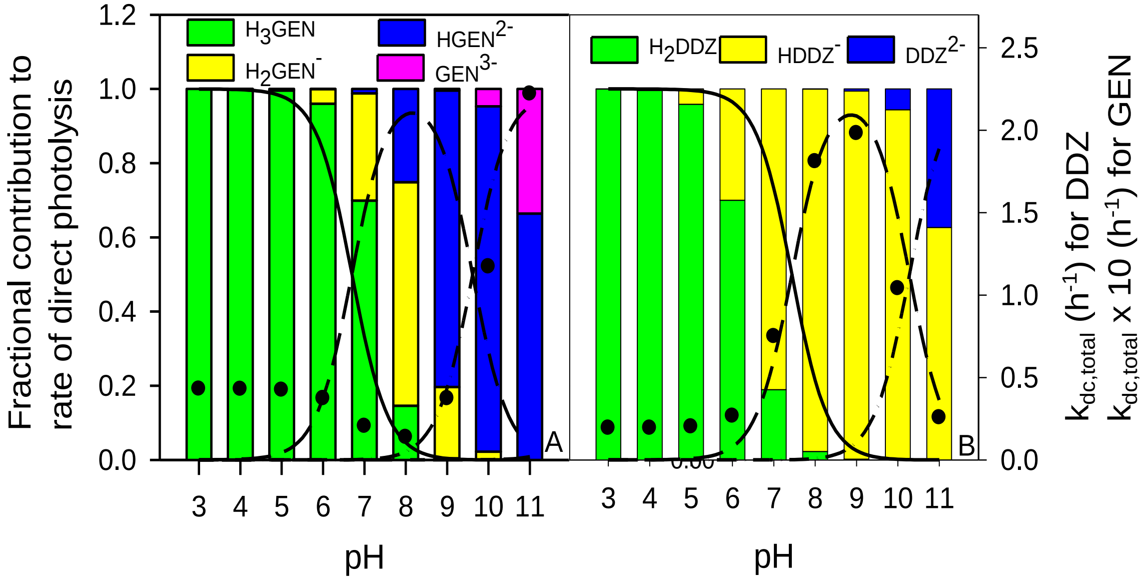


Figure 2-2. Contributions by each protonation state of genistein (A) and daidzein (B) to the overall direct photolysis rate (colored bars) and fractional concentrations of each as a function of pH (H_3GEN or H_2DDZ: solid line; H_2GEN^- or $HDDZ^-$: dashed line; $HGEN^{2-}$ or DDZ^{2-}: dash-dot line; 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 equation 6 and the solar spectrum from ref. 25 for summer 40°N.

Hydroxyl Radical Rate Constant

Concentrations of genistein, daidzein, and acetophenone were monitored during 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 (Fig. A-2), and taking the slope to be $\ln\left(\frac{[C]}{[C_0]}\right)/\ln\left(\frac{[R]}{[R_0]}\right)$ in the equation

$$k_{ROS,C} = \frac{\ln\left(\frac{[C]}{[C_0]}\right)}{\ln\left(\frac{[R]}{[R_0]}\right)} k_{ROS,R} \qquad (2\text{-}9)$$

where $k_{\mathrm{ROS,R}}$ is the rate constant for the reaction between acetophenone and the hydroxyl radical, and $k_{\mathrm{ROS,C}}$ 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 $8.71\pm0.31\times10^{9}$ M^{-1} s^{-1} and $6.94\pm0.09\times10^{9}$ M^{-1} s^{-1}, respectively (Table 2-1; Figure A-2). 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 $\frac{\ln\left(\frac{[C]}{[C_0]}\right)}{\ln\left(\frac{[R]}{[R_0]}\right)}$. The measured rate constants for the reactions of singlet oxygen with genistein or daidzein were $3.57\pm0.08\times10^{7}$ M^{-1} s^{-1} and $1.84\pm0.03\times10^{7}$ M^{-1} s^{-1}, respectively (Table 2-1; Figure A-3). 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, as 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 2-3 for pH 8.5 and in Figure A-1 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 2-3). When added to a pH 8.5 solution of MRW and genistein, isopropanol slightly slowed the loss of genistein ($k_{\text{isopropanol}}/k_{\text{MRW}}$=0.85). Deoxygenation also slightly slowed the reaction ($k_{\text{deoxygenated}}/k_{MRW}$=0.86), but adding sodium azide caused genistein transformation to occur more rapidly ($k_{\text{sodium azide}}/k_{MRW}$=1.46), an unexpected effect seen previously for mefenamic acid.[22] The reaction of genistein in MRW in the presence of sorbic acid ($k_{\text{sorbic acid}}/k_{MRW}$=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 ($k_{\text{sodium azide}}/k_{MRW}$=0.92) and isopropanol ($k_{\text{isopropanol}}/k_{\text{MRW}}$=1). Deoxygenation of the MRW solution slowed the reaction of daidzein substantially ($k_{\text{deoxygenated}}/k_{MRW}$=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 ($k_{\text{sorbic acid}}/k_{MRW}$=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 $^1O_2$ ($k < 5\times10^7$ 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 sodium 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.

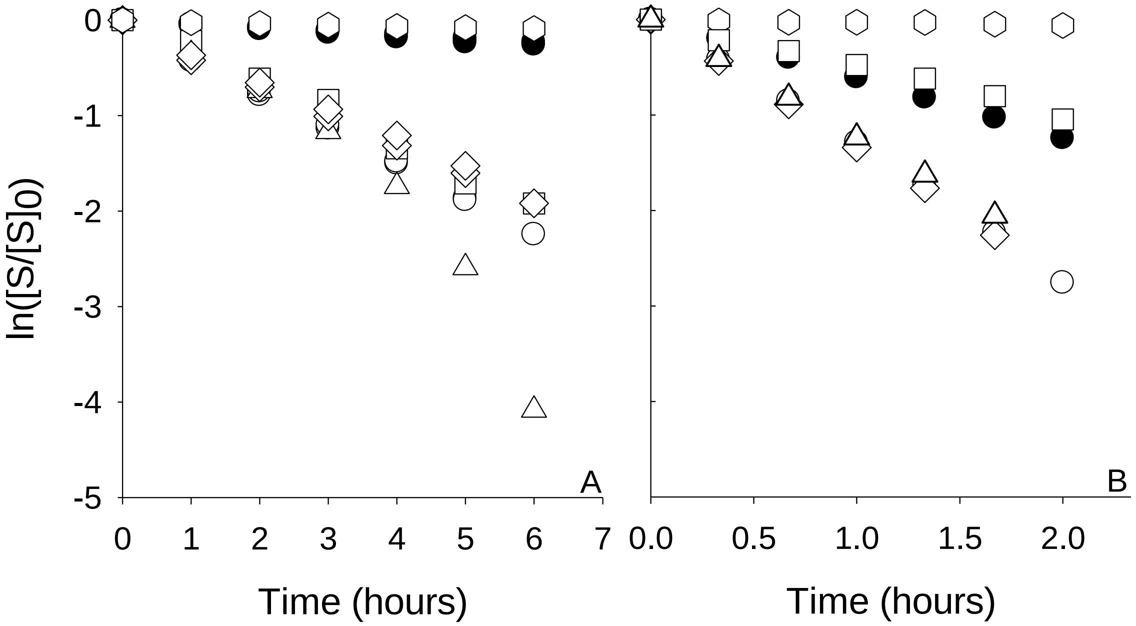


Figure 2-3. Loss of genistein (A) and daidzein (B) in DI water (filled circles), MRW (open circles), deoxygenated MRW (squares), MRW with sodium azide (triangles), MRW with isopropanol (diamonds), and MRW with sorbic acid (hexagons).

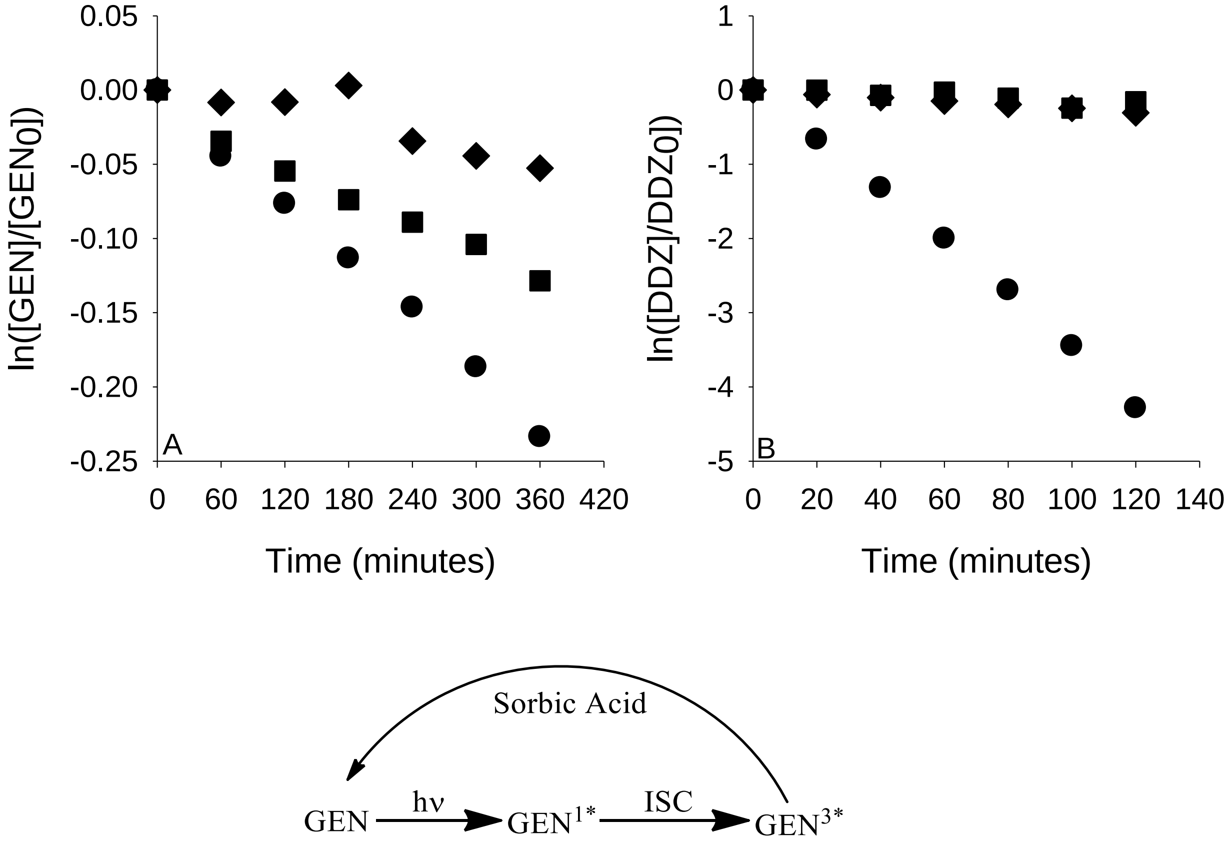


Figure 2-4. Photolysis of genistein (A) and daidzein (B) in DI water with (diamonds) and without (circles) sorbic acid present, and in deoxygenated DI water (squares).

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.

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 2-4 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 2-5). These results indicate that these 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.

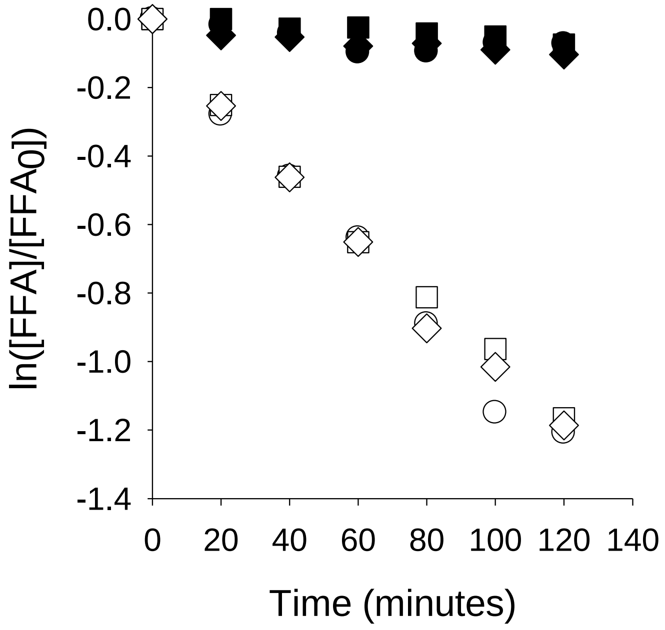


Figure 2-5. Degradation of furfuryl alcohol in the absence (circles) or presence of genistein (diamonds) or daidzein (squares), in DI water (solid) or MRW (outlined).

Predicted Photolysis Half-lives

The half-life of direct photolysis of daidzein in sunlit summer and winter near-surface waters was estimated using equation 2-10.[25]

$$t_{1/2} = \frac{\ln(2)}{\sum_{\lambda} \varepsilon_{\lambda,H_2DDZ}\times L(\lambda)\times \Phi_{H_2DDZ}+\sum_{\lambda} \varepsilon_{\lambda,HDDZ^-}\times L(\lambda)\times \Phi_{HDDZ^-}+\sum_{\lambda} \varepsilon_{\lambda,DDZ^{2-}}\times L(\lambda)\times \Phi_{DDZ^{2-}}} \tag{2-10}$$

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 hours).[25] For daidzein at pH 8.5, the half life was 0.014 days (0.34 hours) in summer and 0.046 days (1.1 hours) in winter. For genistein at pH 8.5, the half life was 1.48 days (35.5 hours) in the summer and 4.91 days (117.8 hours) in the winter.

When samples were irradiated in natural sunlight in MRW at pH 8.7, a half-life of 10 hours was observed for genistein and a half-life of 1.1 hours 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 hours) and 1.84×10^{-6} s^{-1} (half-life of 104.6 hours), 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 2-3B).

For hydroxyl radical typical values are on the order of 10^{-17} to 10^{-15} M.[38] The pseudo-first 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 hours). 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 anti-oxidant.[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 2-3 and 2-4, 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 hours (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 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.

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 Persp.* **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. Heal. A* **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 Persp.* **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. Agr. 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 Sp.* **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. J.; McNeill, K.; Arnold, W. A. 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 Jr, F. E.; 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

(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, N.J., 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.

Chapter 3: Sorption of Phytoestrogens to River Sediment and Model Sorbents

Introduction

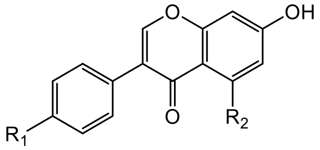
Isoflavones are a class of compounds derived from plants that have been shown to cause reproductive and immunosuppressive effects.[1–3] Isoflavones including genistein, daidzein, and formononetin (Table 3-1) have been detected in municipal wastewater and industrial effluents, drainage waters from pastures and fields, and surface waters at concentrations ranging from 2 ng/L to 151,000 ng/L.[4–13] The photochemical fates of genistein, daidzein, biochanin A, and formononetin have been studied.[14,15] The photolytic half-lives of these isoflavones vary across 2-3 orders of magnitude, depending on conditions including sunlight intensity, pH, quantity and characteristics of dissolved organic matter, and concentrations of reactive oxygen species. While some potential exists for photochemical degradation of isoflavones in the aquatic environment, rates of direct and indirect photolysis do not explain the wide variety of observed isoflavone concentrations in surface waters and effluents.[16] Long photolytic half-lives (up to 120 hours for genistein in deionized water at pH 8.5 and 40° N),[15] suggest that other removal or attenuation mechanisms are worth investigating.

Because the isoflavones have relatively low aqueous solubility (e.g., 0.81 mg/L for genistein, 2.54 mg/L for daidzein), sorption to suspended particles or sediments is a possible mechanism for removal of these compounds from the water column.[17,18] Previous work has addressed the organic carbon-water (K_{oc}) and octanol-water (K_{ow}) partition coefficient values for some isoflavones. The log K_{oc} of daidzein was found to be 3.29, using a Pahokee Peat column.[19] The log K_{ow} values for genistein and daidzein were measured as 3.04 and 2.51, respectively.[20] Experimental values from previous work are approximately within a factor of two or less, compared to predicted values from EPI SUITE.[21] The partitioning constant values suggest that these compounds may sorb to organic-rich phases.

Clay and iron oxide minerals are major components of soils in the intermediate and advanced stages of weathering and related sediments.[22] The sorption of some phenolic compounds to soil minerals at various pH values has been studied. The phenolic, estrogenic compounds estrone, 17α-ethinylestradiol, and bisphenol A all show pH-dependent sorption to montmorillonite but not kaolinite or goethite.[23] Estradiol, a human estrogen shows pH-dependent sorption to goethite but not to kaolinite or montmorillonite.[24] Oxytetracycline, a phenolic antibiotic, shows pH-dependent sorption to goethite, montmorillonite, and kaolinite.[25,26] Thus, the phenolic functional groups on the isoflavones, suggest that pH may influence sorption behavior. The pK_a of the isoflavones values range from 7 to 13,[14,15] indicating that deprotonation will occur at environmentally relevant pH values and must be considered when evaluating sorption to mineral surfaces.

A number of studies of the effects of free, aqueous phytoestrogens on fish have been conducted. Genistein has been shown to affect the gonadal development of male and female Japanese medaka (*Oryzia latipes)* at concentrations from 1 - 1000 μg/L[1] In another study, it was shown to affect the behavior of male fighting fish (*Betta splendens*). Fish exposed to genistein at concentrations from 1 - 1000 μg/L built smaller nests and behaved less aggressively towards a mirror stimulus than unexposed fish.[3] Fighting fish exposed to 1 μg/L and 1000 μg/L produce more dopamine in their forebrain than unexposed fish.[29] Sorption to sediments, however, may not represent a true removal mechanism for isoflavones. Because developing fish in the larval stage are more likely to spend more time in or near sediments, phytoestrogens sorbed to settled particles may pose as great or greater a risk than free, aqueous phytoestrogens. In this study the sorption of isoflavones to mineral surfaces and river sediment is assessed, with the role of pH being critically evaluated.

Table 3-1. Structure and pK_a values of isoflavones and estimates of log K_{oc} and log K_{ow} [a]

Compound		R_1	R_2	pK_a	log K_{ow} [b,d]	log K_{oc} [c]
Genistein		OH	OH	6.7 9.6 13.0[a]	2.84 (3.04)	3.248 3.485
Daidzein		OH	H	7.4 9.9[a]	2.55 (2.51)	2.921 3.367 (3.29)[e]
Formononetin		OCH_3	H	7.0[f]	3.11	3.120 3.065
Biochanin A		OCH_3	OH	7.0 12.3	3.41	3.452 3.183

[a] From ref. 15

[b] Estimated via EPI SUITE[21]

[c] Estimated via EPI SUITE.[21] The first value is from the log K_{ow} method[27] and the second from the MCI method[28]

[d] Experimental values from ref. 19 in parentheses

[e] Experimental values from ref. 20 in parentheses

[f] Estimated to be the same as p$K_{a,1}$ for biochanin A.

Methods

Chemicals. Genistein (96%) and daidzein (95%) were purchased from TCI America. Formononetin (99%) was purchased from Acros Organics. Biochanin A (99.3%) and 3-morpholinopropane-1-sulfonic acid (MOPS, 99.5%) were purchased from Sigma. Sodium chloride (99%), sodium bicarbonate (99.7%), methanol (99.9%) and hydrochloric acid (37%) were purchased from Sigma-Aldrich. Calcium chloride (99%), ammonium acetate (97%), potassium hydroxide (88%), potassium chloride (99.4%), and sodium hydroxide (98.6%) were purchased from Mallinckrodt. Ethanol (91%) was purchased from Fisher. Acetonitrile (100%) was purchased from J.T. Baker.

Goethite was synthesized according to Anschutz and Penn.[30] The goethite has a point of zero net proton charge (pznpc) of 8.47 ($pK_{a1} = 6.7$ and $pK_{a2} = 10.3$) and a surface area of 118.5 ± 2.6 m^2/g as measured by BET and 248 ± 90 m^2/g geometrically.[31] Montmorillonite (SWy-2) was obtained from the Source Clay Minerals Society and prepared homoionically using either sodium chloride or calcium chloride, according to Wang and Arnold.[32] Kaolinite was used as purchased from Riedel de Haen. Sediment was collected from the Mississippi River at the East River Flats in Minneapolis, MN on September 26, 2012. The sediment was dried at 104 °C for 24 hours, then autoclaved three times for 30 minutes each. The fraction of organic matter (f_{om}), determined by loss-on-ignition, is 0.013, giving an approximate f_{oc} of 0.0075, based on a conversion factor of 1.724.[33]

Kaolinite titration. The surface charge of kaolinite was determined by titration. A suspension of 2.5 g kaolinite was made in 50 mL 10 mM KCl. Then, 50 μL 1 M HCl were added, followed by aliquots of 0.78 M KOH. The pK_a of the kaolinite was determined to be 3.45 ($\equiv$Al-OH $\leftrightarrow$ $\equiv$Al-O$^-$). Titration details are in Appendix B.

Sorption Experiments. To describe the sorption of the compounds of interest, sorption isotherms and sorption edges were measured. All experiments were performed in 30 mL serum bottles. The sorbents were kaolinite, montmorillonite, goethite, and Mississippi River sediment. For the clays and river sediment, 0.1 g of sorbent was suspended in 20 mL 10 mM $NaHCO_3$. In all experiments, the pH was adjusted with HCl and NaOH, and the ionic strength was adjusted to the desired values (0.01, 0.11, or 0.51) using NaCl or $CaCl_2$. For sorption edges, the phytoestrogen concentration was 1 mg/L, and the pH ranged from 4 to 12 in half-unit increments. For sorption isotherms, the pH was 6, 8.5, or 11, and the phytoestrogen concentration varied from 0.04 mg/L to 1 mg/L. For goethite sorption edges, 0.1 g of goethite was suspended in 10 mL 10 mM MOPS buffer. The pH ranged from 4.5 to 9.5 in half-unit increments. For goethite isotherms, tested pH values were 5.5, 6.2, 7.1, or 8.2, and 0.2 g of goethite was suspended in 20 mL 10 mM MOPS buffer.[31]

Analytical methods. All batch reactor samples were analyzed by an Agilent 1200 high pressure liquid chromatography (HPLC) system using an isocratic method with 60% acetonitrile and 40% pH 5 10 mM ammonium acetate buffer with 10% acetonitrile. The flow rate was 1 mL/min**.** The HPLC was equipped with a diode array detector and a Supelco Ascentis column (15 cm × 4.6 mm, 5µm particle diameter). Genistein and biochanin A were detected at 259 nm, and formononetin and daidzein were detected at 249 nm.

*Biochanin A pK_a titration.*The pK_a values of biochanin A were determined by spectrophotometric titration (Table 3-1). Solutions of pH values from 4 to 13, in half-unit increments, were adjusted with HCl and NaOH. Then, 0.5 mL of 2.5 mM biochanin A stock in methanol were added to 5 mL pH-adjusted solution. The absorbance of the resulting solutions was analyzed by UV-Vis spectrophotometry on a Shimadzu 1601-UV. The pK_a was determined by fitting equation 3-1 to the absorbances at 284 nm, using Scientist for Windows (v2.1, Micromath),

$$A_\lambda = \frac{A_1[H^+]^2 + A_2[H^+]K_{a1} + A_3K_{a1}K_{a2}}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \tag{3-1}$$

where A_λ is the absorbance at a given wavelength, A_i ($i = 1 – 3$) are the absorbances of the fully protonated, singly deprotonated, and fully deprotonated species of biochanin A, [H$^+$] is –log(pH), and $K_{a1}$and K_{a2} are the acid dissociation constants equal to 10^{-pK_a}. The wavelength 284 nm was chosen by trial and error to maximize the R^2 value of the fit.

Data analysis. Regressions to determine overall solid-water partition coefficient K_d (L/kg) and associated 95% confidence intervals were determined using Microsoft Excel. Data fitting and generation of model parameters was performed in Scientist for Windows (v. 2.1, Micromath). Estimates of log K_{ow} and log K_{oc} were determined using EPI SUITE.[21]

Results and Discussion

EPI SUITE estimates for log K_{oc} and log K_{ow} values are found in Table 3-1. Where experimental literature results are available for comparison, EPI SUITE and the experiments agree reasonably well. One surprising result is that the MCI method for estimating K_{oc} gives a lower value for formononetin than for daidzein, even though the only structural difference is the substitution of a methoxy group for a hydroxy group. To get the MCI estimate, the molecular connectivity is calculated for a molecule, and then correction factors are applied to account for polarity. Comparing the correction factors applied to daidzein and formononetin, a larger correction is taken for formononetin, due to the replacement of a phenol with an aromatic ether, but that replacement causes an increase in molecular connectivity smaller than the increased correction. The experimental value for daidzein is closer to the log K_{ow} method estimate.[20]

Sorption Isotherms. K_d values obtained by sorption isotherms are summarized in Table 3-2. Plots of each isotherm are available in the Appendix B (Figures B-2 – B-6).

Table 3-2. K_d (L/kg) values for phytoestrogens on various sorbents

Sorbent	pH	Genistein	Daidzein	Formononetin	Biochanin A
Goethite	5.5	521.0±189.2[a]	29.6±4.7		
	6.2	397.3±77.7	42.6±6.6		
	7.1	252.8±60.0	27.5±9.5		
	8.2	79.1±13.0		15.7±12.5	
Kaolinite	6.0	113.31±56.7			
	8.5	64.5±15.5	44.2±30.9		1205.5±113.8
	11				
Ca-Montmorillonite	6.0	72.7±10.3			
	8.5	61.1±12.5	53.8±27.6		1309.2±174.1
	11	128.3±25.2			
Na-Montmorillonite	6.0	131.6±37.7			
	8.5	106.6±217.2			80.3±19.4
	11	49.9±6.9			
River Sediment[b]	8.5	57.8±16.2 (7707)	24.1±9.2 (3213)	135.9±60.6 (18120)	

[a] Reported errors are 95% confidence intervals.

[b] Values in parentheses are converted from K_d to K_{oc}, using a conversion factor f_{oc}=0.0075.

Biochanin A shows the highest K_d values for sorption to kaolinite and Ca-montmorillonite. Genistein exhibits 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.[31] Daidzein does not sorb strongly to any of the tested sorbents. When montmorillonite is the sorbent, genistein shows no obvious relationship between K_d and ion composition. For biochanin A and daidzein, however, sorption to Ca-montmorillonite is stronger than to Na-montmorillonite. Converting the K_d values for Mississippi River sediment to log K_{oc} values reveals a log K_{oc} for genistein of 3.88, for daidzein of 3.51, and for formononetin of 4.26. The K_{oc} value for daidzein found in this study is higher than the one predicted by EPI SUITE,[21] but comparable to that found by Schenzel et al.[19] The K_{oc} value for genistein is higher than those predicted by EPI SUITE, but within a factor of 0.6 log units.[21] The K_{oc} value for formononetin is an order of magnitude greater than both values predicted by EPI SUITE, [21] which is likely an underestimate (see above).

Sorption Edges. Because the phytoestrogens have multiple pK_a values, the pH dependence of sorption to the model sorbents was investigated. Genistein and daidzein both clearly display pH-dependent sorption to goethite, both with an observed maximum near pH 7 (Figure 3-1). The sorption edge data were fit to equation 3-2,

$$K_\mathrm{d} = K_1\alpha_{min,o}\alpha_{phyto,0} + K_2\alpha_{min,o}\alpha_{phyto,1} + K_3\alpha_{min,o}\alpha_{phyto,2}+K_4 \tag{3-2}$$

where $K_{1\text{-}4}$ are fitting parameters, $\alpha_{min,0}$ is the fraction of protonated surface sites ($\equiv FeOH_2^+$ for goethite and $\equiv AlOH$ for kaolinite) , and $\alpha_{phyto,0\text{-}2}$ are the fraction of protonated, singly deprotonated, and doubly deprotonated phytoestrogens. with results given in Table 3-3.

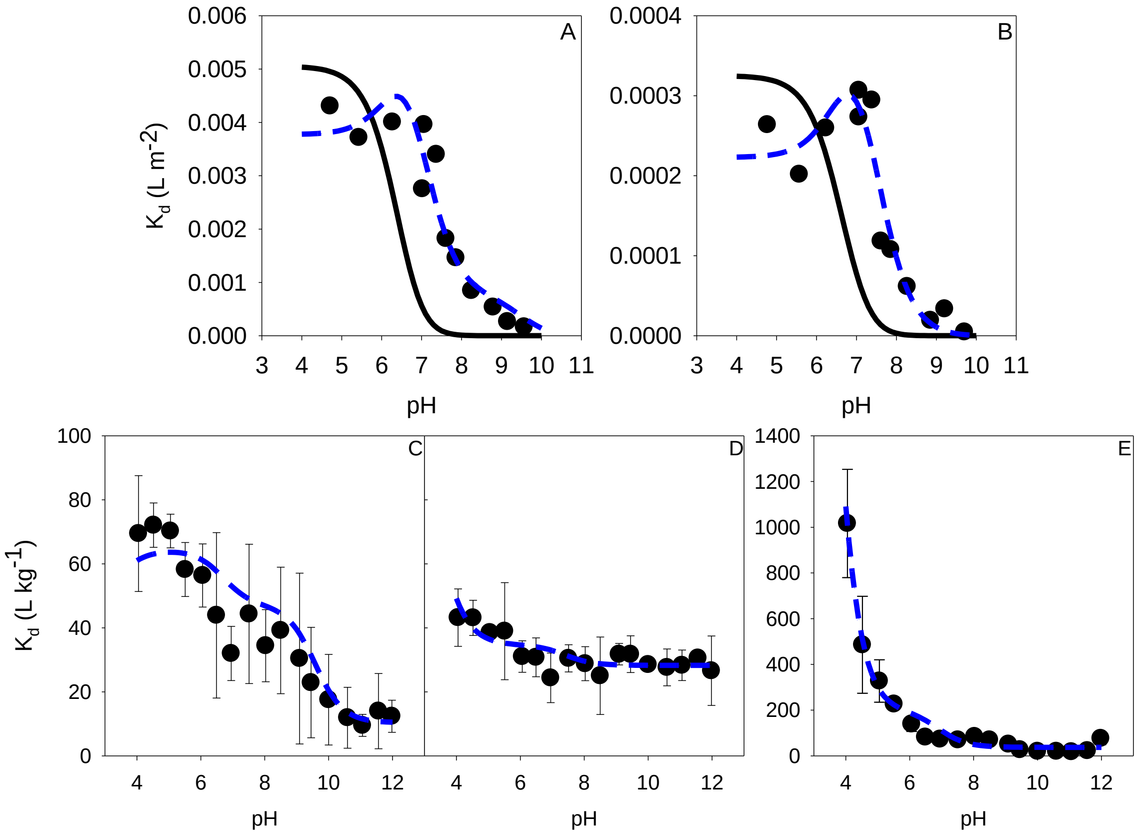


Figure 3-1. Goethite (A, B) and kaolinite (C, D, E) show pH-dependent sorption of genistein (A, C), daidzein (B, D), and biochanin A (E). Error bars represent standard deviations of triplicate data points. Dashed blue lines represent fits to equation 3-2, with results given in Table 3-3. Solid black lines in panels A and B represent fits to equation 3-2, with K_2, K_3, and K_4 set to zero.

The fits suggest that the pH dependence arises mainly from the availability of protonated surface sites, and secondarily from the speciation of the phytoestrogen. Genistein sorbs an order of magnitude more strongly to goethite than does daidzein. The only structural difference between the two molecules is genistein's additional hydroxy group near the oxo group. This structure could form a surface complex with iron, as has been demonstrated for genistein and Fe^{3+} ions in solution.[34]

Table 3-3. Fitted parameters describing sorption of phytoestrogens to goethite and kaolinite.

Goethite	K_1 (L m^{-2})	K_2 (L m^{-2})	K_3 (L m^{-2})	K_4 (L m^{-2})
Genistein	$3.77 \pm 0.88 \times 10^{-3}$	$1.17 \pm 0.31 \times 10^{-2}$	0.524 ± 0.348	0
Daidzein	$2.23 \pm 0.53 \times 10^{-4}$	$2.32 \pm 0.37 \times 10^{-3}$	0	0
Kaolinite	K_1 (L kg^{-1})	K_2 (L kg^{-1})	K_3	K_4 (L kg^{-1})
Genistein	37.8 ± 112.63	$1.03 \pm 0.36 \times 10^{6}$	$5.39 \pm 2.64 \times 10^{7}$	10.34 ± 6.28
Daidzein	67.8 ± 39.9	$5.19 \pm 3.75 \times 10^{5}$	0	28.3 ± 1.9
Biochanin A	$4.49 \pm 0.59 \times 10^{3}$	$50.4 \pm 18.8 \times 10^{5}$	0	37.2 ± 23.5

All tested phytoestrogens exhibited pH-dependent sorption to kaolinite. Data shown represent an average of one trial each of three ionic strengths, which were not appreciably different. Genistein sorption decreases from pH 3 to pH 11. Daidzein and biochanin A both show a rapid decrease in sorption from pH 3 to pH 6, and sorption remains more or less pH-independent at higher pH values. For all three compounds, the most important factor for sorption seems to be the availability of uncharged surface sites. The fully protonated species for each compound has the weakest apparent sorptive interaction with the uncharged kaolinite sites. Because fewer uncharged sites are available at higher pH values, where the more strongly sorbing deprotonated species dominate, the overall sorption decreases with pH. Biochanin A has been shown to complex aluminum in solution, so it is possible that the increased sorption to kaolinite exhibited by biochanin A and genistein compared to daidzein is a result of surface complexation.[34,35]

The K_d values observed for phytoestrogens sorbing to montmorillonite are, on the whole, not as high as those observed for other wastewater-derived contaminants such as the human estrogens and the xenoestrogen bisphenol A, which are assumed to intercalate into montmorillonite layers (Figure 3-2).[23,24] The weaker sorption of phytoestrogens may suggest that they do not intercalate. Montmorillonite does not take on a positive surface charge, and it does not typically have a large number of amphoteric edge groups. So, it is not surprising that no strong pH dependence is observed for sorption of any phytoestrogen to either Na- or Ca-montmorillonite. The sorption of genistein to Na-montmorillonite does show slight pH-dependence, but in contrast to our observations of sorption to kaolinite and goethite, sorption of genistein increases at higher pH. This phenomenon was also observed by Shareef et al. for estradiol.[23] The authors attributed it to fewer edge sites being available as montmorillonite particles flocculated at lower pH values.

An ionic-strength dependence is observed for all three compounds on Ca-montmorillonite, but only a very weak ionic-strength dependence is observed for Na-montmorillonite. This dependence on cation valence has been observed before for xanthan, an anionic polysaccharide, aflatoxin (a mycotoxin), and perfluorinated surfactants.[36–38] For genistein and daidzein, sorption increases with increasing concentration of aqueous calcium. Similar to sorption of oxytetracycline to Chelex-100 resin, this suggests that cation bridging, rather than ion pair interactions, dominates sorption of genistein and daidzein to Ca-montmorillonite.[39]

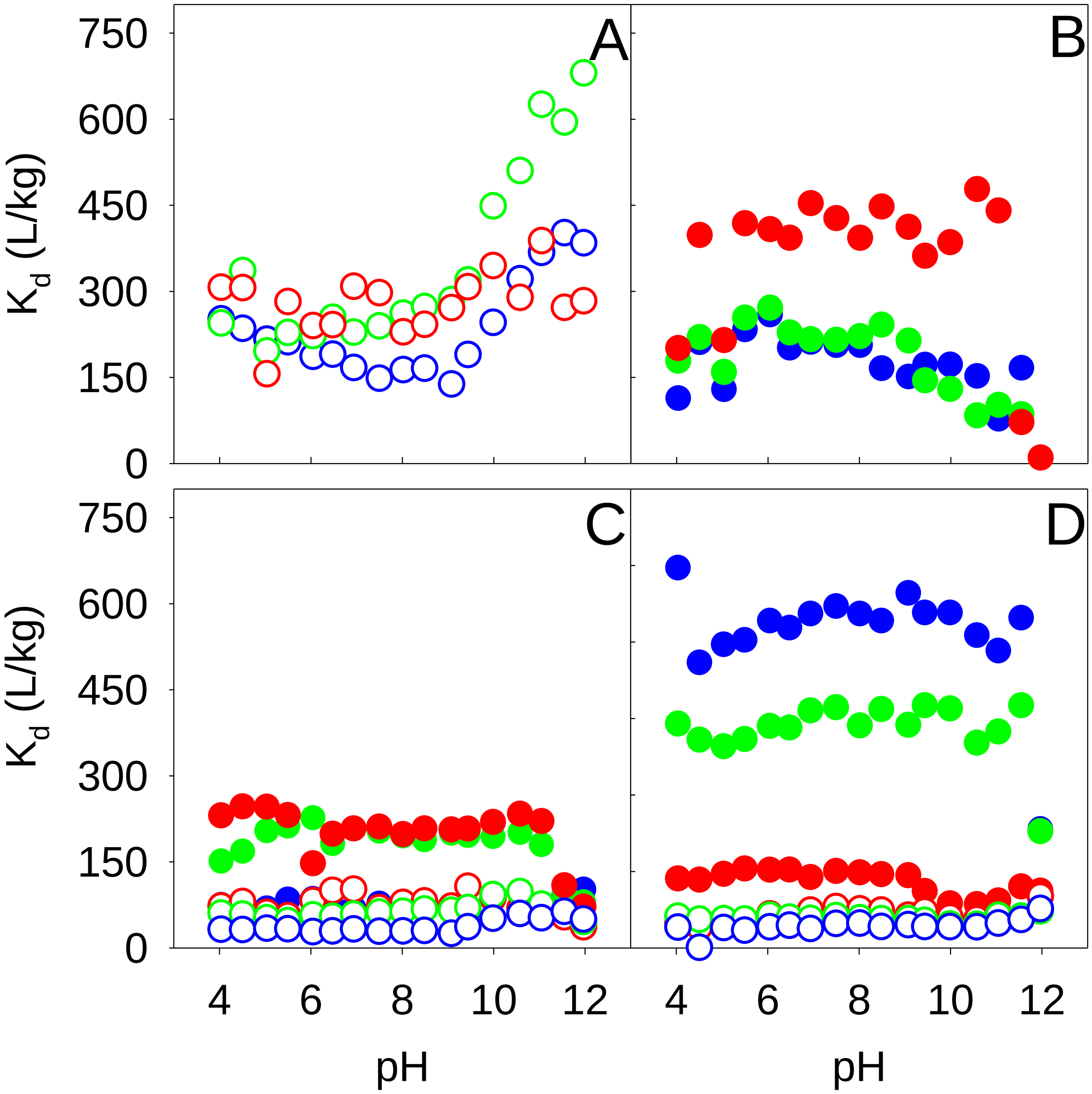


Figure 3-2. Genistein (A, B), daidzein (C), and biochanin A (D) all sorb to montmorillonite, whether it has been exchanged with calcium (solid circles) or sodium (empty circles). Blue points have I=0.01 M, green points have I=0.11 M, and red points have I=0.51 M.

Decreasing sorption with increasing ionic strength usually indicates the formation of an outer-sphere surface complex, which becomes less feasible for the sorbate of interest as electrolyte ions cover more of the available surface area.[40] In general, sediments with large fractions of clay minerals will not be strongly influenced by pH under environmentally relevant conditions. In systems with variable ionic strength, such as estuaries and northern aquatic systems receiving large amounts of road salt from run-off, sorption to montmorillonite and other 2:1 clay minerals may vary with salt inputs.

Compared to traditional persistent organic pollutants, isoflavones do not sorb especially strongly to clay minerals, iron oxides, or natural sediments. Under circumstances with high concentration or frequent discharges of isoflavones to natural waters, however, sorption to settling particles will play a role in the attenuation of the discharge. The role of sorption in the total attenuation 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.

Compared to other contaminants of emerging concern, such as human and synthetic estrogens, bisphenol A, and the tetracyclines, sorption is a similarly or slightly less important attenuation process for isoflavones. Mestranol, a derivative of 17α-ethinyl estradiol (EE2) replacing the phenolic group with a methoxy group, has a K_d value for sorption to montmorillonite of over 1200 L/kg from pH 4-12, while that for EE2 increases from 180 L/kg at pH 4 to 750 L/kg at pH 10. The sorption of the human estrogen estrone (E1) to montmorillonite is pH-dependent, increasing from 60 at pH 4 to 200 at pH 10. The xenoestrogen bisphenol A sorbs to montmorillonite, with K_d increasing from 750 at pH 4 to 1600 at pH 10. Bisphenol A, E1, and EE2 all show pH-independent sorption to goethite, with K_d values of 170, 100, and 130 L/kg, respectively, and weak, pH-independent sorption to kaolinite, with K_d values less than 50 L/kg.[23] Of the estrogens evaluated by Shareef et al., none has a pK_a below 9, which likely explains the opposite findings of pH-dependent sorption on the three minerals investigated compared to the isoflavones, all of which have pK_a values near 7.[23] Oxytetracycline, a phenolic antibiotic, shows a different edge shape for sorption to goethite, compared to genistein and daidzein, because of its ability to form a cation or zwitterion. At its maximum, oxytetracycline has a K_d near 0.01 L/m^2, while the maximum for genistein is only 0.004 L/m^2 and that for daidzein is only 0.0003 L/m^2.[26] Compared to the sorption of oxytetracycline on clays, the sorption of isoflavones is very weak. Oxytetracycline, however, sorbs less to Na-montmorillonite at greater ionic strength, like biochanin A and Ca-montmorillonite. Figueroa and Mackay attribute the ionic strength effect to cation exchange, which is not a possibility for the acidic isoflavones.[25]

Given their generally low K_d values and good fits to the presented model (eq. 3-2), it seems likely that the isoflavones are sorbing to goethite and kaolinite by electrostatic interactions. Genistein and biochanin A have both been shown to form complexes with Al^{3+} and Fe^{3+} ions in solution, so surface complexation may also be involved. For montmorillonite, it seems that genistein and daidzein sorb by cation bridging or hydrophobic partitioning, while the ionic strength effect observed for biochanin A suggests an outer-sphere surface complex to Ca-montmorillonite.

Using data from the isotherms presented here, the sorbed fraction f_s of genistein and daidzein in a hypothetical pond were calculated according to the equation

$$f_s = 1 - 1/(1 + r_{sw}(f_{oc}K_{oc} + f_{kao}K_{kao} + f_{gt}K_{gt} + f_{NaM}K_{NaM}) \qquad (3\text{-}3)$$

where r_{sw} is the solid-water ratio, or concentration of suspended sediments in kg/L, f_{oc} is the fraction of organic carbon, f_{kao} is the fraction of kaolinite, f_{gt} is the fraction of goethite, f_{NaM} is the fraction of Na-Montmorillonite, and each K represents the distribution coefficient presented in Table 3-2.[41] 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 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 sedimentation rate k_s^* equal to the settling velocity v_s times the fraction sorbed f_s, and a settling velocity of 0.5 day^{-1}, 1.9% of the genistein and 0.8% of the daidzein would settle with particles per day. Therefore, sorption to particles may be important for very slow-moving aquatic systems receiving inputs of isoflavones.

References

(1) 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 Persp.* **2003**, *111*, 1158–1163.

(2) 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.

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

(4) 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. Agr. Food Chem.* **2007**, *55*, 8339–8345.

(5) Hoerger, C. C.; Wettstein, F. E.; Bachmann, H. J.; Hungerbühler, K.; Bucheli, T. D. Occurrence and mass balance of isoflavones on an experimental grassland field. *Environ. Sci. Technol.* **2011**, *45*, 6752–60.

(6) 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.

(7) Hoerger, C. C.; Schenzel, J.; Strobel, B. W.; Bucheli, T. D. Analysis of selected phytotoxins and mycotoxins in environmental samples. *Anal. Bioanal. Chem.* **2009**, *395*, 1261–89.

(8) 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.

(9) Ribeiro, C.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. *Arch. Environ. Con. Tox.* **2009**, *56*, 1–11.

(10) Ribeiro, C.; Pardal, M. A.; Tiritan, M. E.; Rocha, E.; Margalho, R. M.; Rocha, M. J. Spatial distribution and quantification of endocrine-disrupting chemicals in Sado River estuary, Portugal. *Environ. Monit. Assess.* **2009**, *159*, 415–27.

(11) 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.

(12) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P.; Samperi, R.; Laganà, A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**, *531*, 229–237.

(13) 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 Sp.* **2006**, *20*, 2411–2418.

(14) Felcyn, J. R.; Davis, J. C. C.; Tran, L. H.; Berude, J. C.; Latch, D. E. Aquatic photochemistry of isoflavone phytoestrogens: Degradation kinetics and pathways. *Environ. Sci. Technol.* **2012**. *46*, 6698-6704.

(15) Kelly, M.; Arnold, W. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environ. Sci. Technol.* **2012**, *46*, 5396–5403.

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

(17) Stancanelli, R.; Mazzaglia, A.; Tommasini, S.; Calabrò, M. L.; Villari, V.; Guardo, M.; Ficarra, P.; Ficarra, R. The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: a spectroscopic investigation with implications in the pharmaceutical analysis. *J. Pharmaceut. Med.* **2007**, *44*, 980–4.

(18) Han, H.-K.; Lee, B.-J.; Lee, H.-K. Enhanced dissolution and bioavailability of biochanin A via the preparation of solid dispersion: in vitro and in vivo evaluation. *Int. J. Pharm.* **2011**, *415*, 89–94.

(19) Schenzel, J.; Goss, K.-U.; Schwarzenbach, R. P.; Bucheli, T. D.; Droge, S. T. J. Experimentally determined soil organic matter-water sorption coefficients for different classes of natural toxins and comparison with estimated numbers. *Environ. Sci. Technol.* **2012**, *46*, 6118–6126.

(20) Rothwell, J.; Day, A. Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. *J. Agr. Food Chem.* **2005**, *53*, 4355–4360.

(21) US EPA Estimation Programs Interface Suite(TM) for Microsoft(R) Windows, v 4.1 **2013**.

(22) Sposito, G. *The Chemistry of Soils*; Oxford University Press, USA, 2008; p. 329.

(23) Shareef, A.; Angove, M. J.; Wells, J. D.; Johnson, B. B. Sorption of bisphenol A, 17alpha-ethynylestradiol and estrone to mineral surfaces. *J. Colloid Interf. Sci.* **2006**, *297*, 62–9.

(24) Van Emmerik, T.; Angove, M. J.; Johnson, B. B.; Wells, J. D.; Fernandes, M. B. Sorption of 17 [beta]-estradiol onto selected soil minerals. *J. Colloid Interf. Sci.* **2003**, *266*, 33–39.

(25) Figueroa, R. A.; Leonard, A.; MacKay, A. A. Modeling Tetracycline Antibiotic Sorption to Clays. *Environ. Sci. Technol.* **2004**, *38*, 476–483.

(26) Figueroa, R. A.; MacKay, A. A. Sorption of Oxytetracycline to Iron Oxides and Iron Oxide-Rich Soils. *Environ. Sci. Technol.* **2005**, *39*, 6664–6671.

(27) Doucette, W. Soil and sediment sorption coefficients. *Handbook of Property Estimation Methods for Chemicals: Environmental and Health Sciences* **2000**.

(28) Meylan, W.; Howard, P. H.; Boethling, R. S. Molecular topology/fragment contribution method for predicting soil sorption coefficients. *Environ. Sci. Technol.* **1992**, *26*, 1560–1567.

(29) Clotfelter, E. D.; McNitt, M. M.; Carpenter, R. E.; Summers, C. H. Modulation of monoamine neurotransmitters in fighting fish Betta splendens exposed to waterborne phytoestrogens. *Fish Physiol. Biochem.* **2010**, *36*, 933–43.

(30) Anschutz, A.; Penn, R. L. Reduction of crystalline iron(III) oxyhydroxides using hydroquinone: Influence of phase and particle size. *Geochem. T.* **2005**, *6*, 60-66.

(31) Overgaard, C. G. Adsorption of the phytoestrogens, genistein and daidzein, onto the iron oxide-hydroxide goethite **2012**. University of Stavanger.

(32) Wang, S.; Arnold, W. A. Abiotic reduction of dinitroaniline herbicides. *Water Res.* **2003**, *37*, 4191–201.

(33) Lyman, W.; Reehl, W.; Rosenblatt, D. *Handbook of chemical property estimation methods;* American Chemical Society: Washington, D.C., **1990**.

(34) Dowling, S.; Regan, F.; Hughes, H. The characterisation of structural and antioxidant properties of isoflavone metal chelates. *Journal of inorganic biochemistry* **2010**, *104*, 1091–8.

(35) Deng, H.; Van Berkel, G. J. Electrospray mass spectrometry and UV/visible spectrophotometry studies of aluminum(III)-flavonoid complexes. *J. Mass Spectrom.* **1998**, *33*, 1080–1087.

(36) Deng, Y.; Liu, L.; Luisa Barrientos Velázquez, A.; Dixon, J. B. The Determinative Role of the Exchange Cation and Layer-Charge Density of Smectite on Aflatoxin Adsorption. *Clay Clay Min.* **2012**, *60*, 374–386.

(37) Higgins, C. P.; Luthy, R. G. Sorption of Perfluorinated Surfactants on Sediments. *Environ. Sci. Technol.* **2006**, *40*, 7251–7256.

(38) Dontsova, K. M.; Bigham, J. M. Anionic Polysaccharide Sorption by Clay Minerals. *Soil Sci. Soc. Am. J.* **2005**, *69*, 1026-1035.

(39) MacKay, A. A.; Canterbury, B. Oxytetracycline sorption to organic matter by metal-bridging. *J. Environ. Qual.* **2005**, *34*, 1964–71.

(40) Lützenkirchen, J. Ionic Strength Effects on Cation Sorption to Oxides: Macroscopic Observations and Their Significance in Microscopic Interpretation. *J. Colloid Interf. Sci.* **1997**, *195*, 149–155.

(41) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

Chapter 4. Microbiological degradation of phytoestrogens and the response of fathead minnows to degradate exposure

Introduction

Cultivated and uncultivated plants contain varying concentrations of phytoestrogens, a class of plant-produced hormonally 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 produced in the highest concentrations from cultivated plants and tend to be the most estrogenic in this compound class.[1-10] Indeed, numerous studies have linked phytoestrogen exposure in fish to wide ranging reproductive, developmental or behavioral effects at levels as low as 1 µg/L. [6,11-14]

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 nineteen industrial effluents, eight contained high phytoestrogen concentrations, dominated by genistein and daidzein: biodiesel (1.3-22.5 µg/L), soy milk (250 µg/L), ethanol (4.7 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).[15] Effluents from pulp and paper mills have also been found to contain high concentrations of phytoestrogens, with one study measuring genistein concentrations in pulp and paper mill effluent at 10.1 µg/L.[8,16] 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.[17] Studies conducted on municipal WWTP effluents have detected the presence of phytoestrogens at a range of concentrations.[15,18-20] 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 [15,21-23]

Although the details of phytoestrogen degradation within receiving waters have yet to be examined, degradation across municipal WWTPs has been observed.[15,18,23] 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. If estrogenicity decreases following degradation, as has been observed with the microbiological degradation of steroidal estrogens, the implementation of strategies to control phytoestrogen discharge and impact should be straightforward (*i.e*., wastewater treatment via microbiological degradation).[24]

Given the rapid expansion of plant processing 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 and was also assessed in the presence of an inhibitor of nitrification. Finally, organismal response (larval and sexually mature fathead minnows) was quantified following exposure to phytoestrogen degradates. Larvae were exposed to the degradates of the commonly detected phytoestrogens genistein, daidzein, and formononetin, singly and in a mixture. Adults were exposed to the degradation products of the mixture of genistein, daidzein, and formononetin. Exposures to the degradates of the single compounds were not conducted with adult minnows because of the more involved experimental set-up and expected presence of phytoestrogen mixtures in discharges.[15,25] Together, results from these three objectives allow for a much clearer assessment of phytoestrogen risk to an ecologically relevant fish species (the fathead minnow) based on genistein and daidzein persistence in a variety of surface waters and the biological effects of their degradates.

Methods

Chemicals

Genistein (96%) and daidzein (95%) were purchased from TCI America. Formononetin (99%) was purchased from Acros Organics. Biochanin A (99.3%) was purchased from Sigma. Sodium azide (99%) was purchased from Janssen Chimica. Methanol (99.9%) was purchased from Sigma-Aldrich. Ammonium acetate (97%) was purchased from Mallinckrodt. Acetonitrile (100%) was purchased from J.T. Baker.

Surface water collection. Water from the Minnesota River and Okabena Creek was collected 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 wastewater treatment plant outfalls, respectively. Surface water from the Mississippi River was collected in the same fashion, at the East River Flats (Minneapolis, MN), on 6/26/13. 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 within two weeks of collection.

Genistein and daidzein biodegradation kinetics. 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 give the 120 mL river water an initial 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) into HPLC vials and directly analyzed by HPLC without preconcentration. Three control reactors containing both genistein and daidzein and 50 mM sodium azide were constructed in the same manner as experimental reactors.

Genistein biodegradation under different environmental conditions. Batch reactors were set-up in triplicate in sterilized 4-L Erlenmeyer flasks capped with gas permeable sponge stoppers. Reactors were covered with foil to prevent loss via photolysis.[26] Oxygen was supplied via stirring and headspace entrainment. Water (4 L) collected from the Minnesota River or Okabena Creek was allowed to equilibrate to the desired temperature (20 or 10°C), after which it was added to the reactors. Genistein was added to the reactors to begin the experiments in one of two ways, depending on the desired final concentration: to reach an initial concentration of 0.5 μg/L genistein, an aqueous stock solution was used (800 μg/L, pH 11); to reach an initial concentration of 100 μg/L genistein, a methanolic (100 μg/mL) stock was added as described above. Initial genistein concentrations were: Minnesota River water collected on 5/14/12 and 6/6/12: 0.5 μg/L genistein, collected on 11/8/11 and 5/14/12: 100 μg/L genistein; Okabena Creek water collected on 6/6/12: 0.5 μg/L genistein, collected on 11/8/11: 100 μg/L genistein. Negative controls were set-up in triplicate and amended with 50 mM sodium azide.

Samples were withdrawn from the reactors over time and analyzed for genistein concentration. In those reactors receiving 0.5 μg/L genistein, approximately 200-mL samples were withdrawn over time, amended with d_3-genistein (surrogate, Cambridge Isotopes), concentrated via SPE, and analyzed by liquid chromatography-mass spectrometry (LC-MS). In the reactors receiving 100 μg/L genistein containing the water collected on 11/8/11, 100-mL samples were withdrawn over time, amended with d_3-genistein, concentrated via SPE, and analyzed by HPLC. In the reactors receiving 100 μg/L genistein containing the water collected on 5/14/12, 0.5-mL samples were withdrawn over time, syringe-filtered with a glass fiber filter, amended with the d_3-

genistein surrogate, and analyzed directly by LC-MS. Periodic reactor samples were taken for optical density (a measure of biomass growth) and dissolved organic carbon determination.

Genistein biodegradation under nitrifying conditions. Activated sludge was collected from the Metropolitan Wastewater Treatment Plant 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 (Appendix C, Table C1) 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 and a 12-hour hydraulic residence time. The biomass from this reactor was rinsed and then used to inoculate six additional 4-L Erlenmeyer flasks to 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. DO was maintained above 4 mg/L using air-stones. 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), amended with d_3-genistein, concentrated via SPE, and analyzed by LC-MS (described below). Total ammonium/ammonia, nitrate, and nitrite, and suspended solids were also measured periodically (described below).

Sample preparation for phytoestrogen analysis. For the kinetic experiments using Mississippi River water, the SPE cartridges (1000 g C_{18}, 6 mL, Restek) were conditioned with two column volumes of methanol and two column volumes of water before the samples were loaded drop-wise onto the cartridges. The cartridges were rinsed with one column volume of water before elution by gravity with methanol. For the Minnesota River and Okabena Creek experiments, 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) 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. The acetone was blown down to approximately 0.25 mL using a gentle stream of nitrogen (UHP/Zero-grade). All solvents used for sample preparation were HPLC-grade.

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 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. A sample injection volume of 35 µL was used together with a 1 mL/min flow rate. 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). The method limit of quantification (LOQ) was determined be 4.43 µg/L for genistein, 3.53 µg/L for daidzein and 2.79 µg/L for formononetin. 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. Relative recovery for the SPE was tested and found to be 30.0% ± 1.1%, 41.1% ± 7.7%, and 30.4% ± 3.8% for daidzein, formononetin, and genistein, respectively.

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 and measured three times; the average value was recorded. Glass fiber filters used in SS, VSS and DOC analysis were pre-combusted at 500°C for 30 min to remove water and volatile organics and stored in a desiccator until use. SS and VSS were measured according to Standard Method 2540D and 2540E, respectively.[27] Samples (20 mL) were analyzed for DOC by first filtering 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.

Ion analysis. Total ammonium/ammonia concentration was determined using a Thermo Scientific Orion Ammonia Specific Electrode. GFF-filtered samples (2 mL) were adjusted to pH 11 using 100 µL of Ammonia ISA Solution (HACH). The probe was placed in the sample and continuously mixed until the conductivity stabilized. An external calibration curve was used to quantify ammonium/ammonia. Nitrate and nitrite concentrations were determined using a Metrohm 761 ion chromatograph using a Metrohm 766 sample processor and IC Net software.

Laboratory exposure experiments. To assess the biological effects of biologically degraded phytoestrogens (singly and in a mixture) at concentrations reflecting environmental occurrence, larval and 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 the microbiological products of three single phytoestrogens (larvae only), genistein, daidzein, and formononetin and of their mixture (larvae and adults). 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 five treatment groups: degraded daidzein, degraded formononetin, degraded genistein, degraded mixture (mixture high from earlier adult exposures), and a lake water control incubated similarly to the degraded phytoestrogen samples, providing 150 larvae tested (15 beakers, 3 per treatment). 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) twice daily following established US EPA culturing procedures.[28] Composite water samples (200 mL from each of the three beakers in a treatment) were taken every four days to confirm the lack of parent phytoestrogens in the samples. Samples were acidified in 1L amber bottles and kept at 4°C until analysis.

Following exposure, predator avoidance performance was assessed following previously published methods.[29-31] 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, degraded genistein, daidzein, formononetin, mixture, control, 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 also quantified. All data were digitized using morphometric software (NIH ImageJ).

Adult fathead minnow exposures to phytoestrogens and their degradates. Adult fathead minnows were exposed to one of three treatments: a mixture of 1000 ng/L each daidzein, genistein, and formononetin, an ethanol control, and the effluent from an aerobic biological reactor (described below) degrading a mixture of daidzein, genistein, and formononetin, diluted to reach what would have been 1000 ng/L each if no degradation had taken place. Exposure followed a 21-day exposure regime as described below.

A 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 a modified synthetic sewage media (Tables C2-3) and 500 µg/L of each 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 suspended solids (data not shown). Three water samples were collected to verify that parent phytoestrogens were completely degraded before entering the dilution chamber at the aquaria interface (Table C4). Samples were acidified, amended with d_3-genistein and d_3-daidzein surrogates, concentrated by SPE, and analyzed on the LC-MS (described above).

Mature (approximately six months old) fathead minnows were obtained from a laboratory fish supplier (Environmental Consulting and Testing Inc). Each treatment contained four aquaria with randomized assignment of 10 males per 35L aquarium (2 aquaria) or 10 females (2 aquaria). Fish maintenance followed established protocols.[28] The flow-through exposure system continuously pumped the appropriate amount of a concentrated phytoestrogen stock solution or the effluent from the bioreactor into a temperature-adjusted flow of well water supplied by a dedicated in-house well. The system provided flow rates of 200 mL/min per aquarium allowing for approximately eight exchanges per day. All environmental parameters during the exposure experiment were maintained constant as follows: 21 ± 1°C; 5.5 ± 1.0 mg/L dissolved oxygen; 240 ppm $CaCO_3$ alkalinity; 0.95 ± 0.02 mS/cm conductivity; and 8.1 ± 0.1 pH. Abiotic parameters were monitored using a YSI-556 unit and Hach 5-in-1 test strips. Ammonia was measured weekly using a Hach low-range test kit (mg/L). Water temperature was recorded using Hobo data loggers (Onset Computer Corp.) set to record temperature at 10-minute intervals.

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), total and standard length (mm) were taken prior to dissection to calculate body condition factor = (total mass/(total length^3))*100000.[32] 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 massed (g) and stored in 10% buffered formalin. Tissues were dehydrated using a Leica 1050 Automated Tissue Processor and embedded using a Tissue-Tek 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) followed protocols established by the US EPA.[33]

Whole blood was taken from the caudal region using heparinized capillary tubes. A competitive ELISA (polyclonal fathead minnow antibody) was used to quantify vitellogenin concentrations by incorporating a species-validated anti-vitellogenin antibody and purified vitellogenin as standards following previously published protocols.[34] 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 4800 µg/mL.

The remaining fish were transferred as single-treatment pairs of one male and one female fish to 4L 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 10cm 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 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.[35] 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 60s 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 between across testing events for statistical analysis.

Statistical analysis. Stata 10.1 (StataCorp) 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:

$$C(t) = C_0 e^{be^{ct}} \tag{4-1}$$

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 MN 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 in surface water data in order to assess statistical significance between two data sets. Data from the minnow exposure experiments were tested for normality using a Kolgomorov-Smirnov test. For the larval exposure experiment, beakers within treatment 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 vitellogenin 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 4.01, GraphPad Software Inc.). 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 and Discussion

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 4-1. Degradation was rapid after a variable lag period (Appendix C, Figures C1-C3) and although the rate increased with concentration, it did not level off at the higher initial concentrations tested. This suggests that 100 μg/L is 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 4-1).

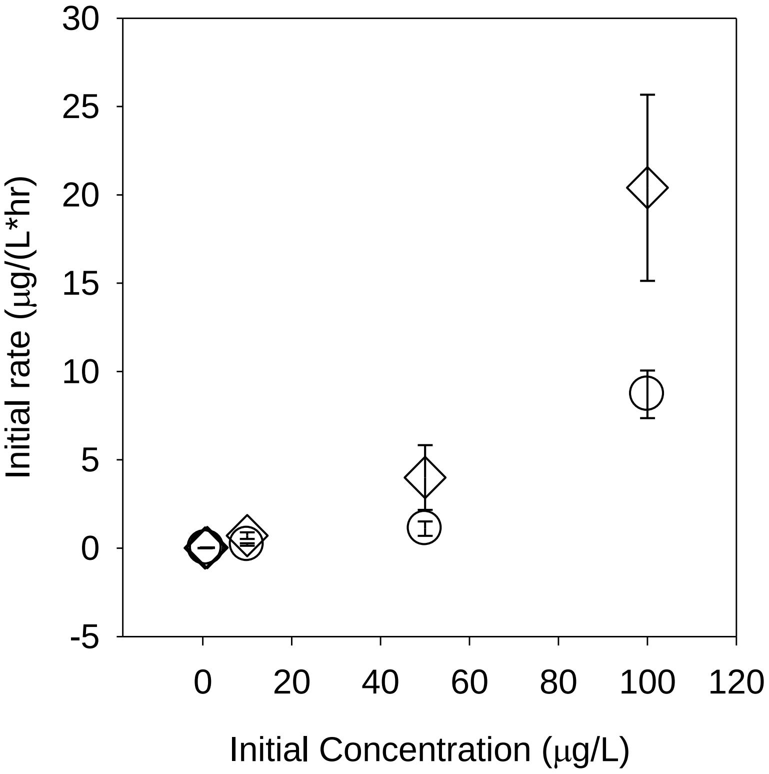


Figure 4-1. Initial degradation rates of genistein (diamonds) and daidzein (circles) (given as the first order rate constant multiplied by initial concentration) at different initial concentrations in Mississippi River water.

Data were also fit to zero- and first-order models, which both fit the lowest two initial concentrations of genistein (0.5 and 1.0 μg/L) better than the Gompertz model (Table 4-1), perhaps simply because of the scatter in the data and the larger number of parameters to be fit by the Gompertz model.

Table 4-1. Fitting parameters describing degradation of genistein and daidzein

	Gompertz c		First-order		Zero-order	
Genistein						
C_0 (mg/L)	c ($\times 10^{-1}$ h^{-1})	R^2	k ($\times$ 10^{-2} h^{-1})	R^2	k (μg L^{-1}hr^{-1})	R^2
100[A]	3.58 ± 0.63	0.99	9.23 ± 5.27	0.94	8.95 ± 0.91	1.0
100[B]	3.63 ± 4.32	0.99	23.1 ± 7.5		6.08 ± 0.77	
100[C]	5.42 ± 3.26	0.99	9.37 ± 23.4		2.86 ± 1.12	
100[D]	3.48 ±1.61	0.99	12.9 ± 18.3		2.92 ± 2.07	
50[A]	3.92 ± 0.14	0.99	8.00 ± 3.66	0.90	2.82 ± 1.5	0.95
10[A]	2.16 ± 0.13	0.99	7.11 ± 1.88	0.96	0.271 ± 0.048	0.98
1[A]	2.81 ± 0.58	0.75	3.59 ± 1.53	0.90	0.015 ± 0.007	0.86
0.5[A]	2.59 ± 17.4	0.49	1.27 ± 1.88	0.80	0.0037 ± 0.0058	0.79
0.5[B]			2.24 ± 0.13		0.00488 ± 0.00056	
0.5[E]			2.11 ± 0.070		0.00796 ± 0.00179	
0.5[F]			1.76 ± 0.017		0.00207 ± 0.00063	
0.5[G]			3.39 ± 1.76		0.00700 ± 0.00242	
Daidzein						
100[A]	2.56 ± 0.09	0.99	8.71 ± 1.35	1.00	6.49 ± 0.97	1.0
50[A]	0.759 ± 0.465	0.96	2.22 ± 0.81	0.92	0.814 ± 0.164	0.95
10[A]	0.816 ± .380	0.98	2.11 ± 0.66	0.94	0.139 ± 0.024	0.97
1[A]	0.380 ± 0.262	0.98	1.32 ± 0.44	0.97	0.00952 ± 0.00231	0.97
0.5[A]	0.719 ± 0.062	0.96	1.33 ± 0.77	0.92	0.00539 ± 0.00235	0.93

[A] Mississippi River, 6-26-13, 20°
[B] Minnesota River, 5-14-12, 20°
[C] Minnesota River, 11-8-11, 20°
[D] Okabena Creek, 11-8-11, 20°
[E] Okabena Creek, 6-6-12, 20°
[F] Minnesota River, 6-6-12, 10°
[G] Minnesota River, 6-6-12, 20°

Genistein degradation as a function of environmental conditions

Additional experiments were performed, focusing on genistein, to better gauge how surface water source, incubation temperature, and time (i.e., season) of surface water collection affected genistein degradation kinetics (Figure 4-2).

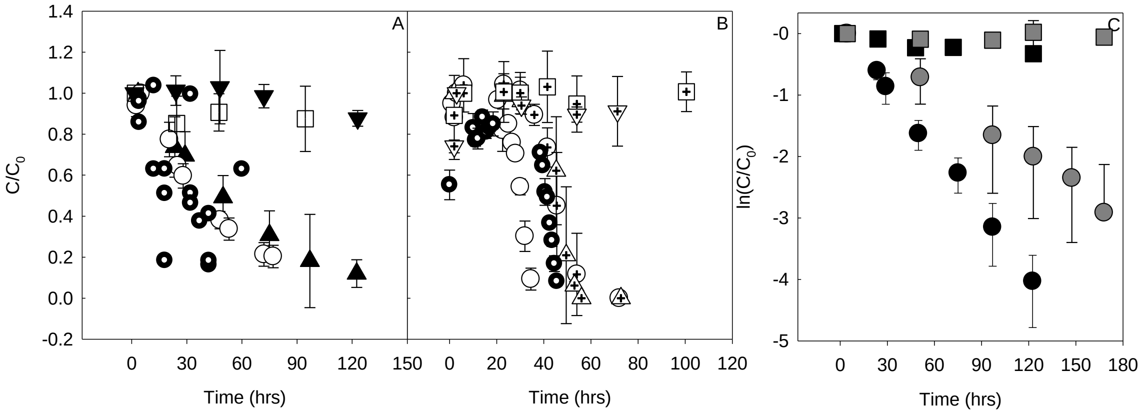


Figure 4-2. Degradation of genistein at an initial concentration of 0.5 μg/L (A, C) or 100 μg/L (B) in water collected on 11-8-11 (cross-hairs), 5-14-12 (white), 6-16-12 (black), and 6-26-13 (rings) from the Mississippi River (rings experimental, controls in SI) Okabena Creek (up triangles experimental, down triangles controls) or the Minnesota River (circles experimental, squares control) The grey points represents samples from 6-6-12 incubated at 10° C instead of 20° C.

Rates of biodegradation depended on initial concentration in all of the water sources examined (Figures 4-1 and 4-2) and also depended on temperature and time of surface water collection (i.e., season of collection). 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 first-order degradation rate coefficient in Minnesota River samples (collected 6/6/12, 0.5 μg/L initial concentration) (Figure 4-2). With the same water source (Minnesota River), the time of surface water collection also affected genistein degradation rates, with significantly higher genistein degradation rate coefficients calculated for water samples collected during warmer and presumably more biologically-active months (i.e., p=0.0195 for samples collected 6/6/12 as compared to 5/14/12 and p=0.0258 for samples collected 5/14/12 as compared to 11/8/11) (Figure 4-2, Table 4-1). Perhaps surprisingly, comparing data from samples collected on the same day, genistein first-order degradation rates depended on the source of the surface water (Okabena Creek versus the Minnesota River) at low (0.5 µg/L, p=0.0291) but not high (100 µg/L, p=0.0914) initial concentrations (Table 4-1). Genistein degradation in Mississippi River water is shown in Figure 4-2 but was not statistically compared to Minnesota River or Okabena Creek data as a result of the different experimental set-ups used. Nevertheless, it appears that genistein was degraded at a rate of the same magnitude in Mississippi River water as in Okabena Creek or Minnesota River water. Overall abiotic losses of genistein were low (Figure 4-2C, Figures C1-3). Again, 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.[36] If nitrifying bacteria are also responsible for genistein biodegradation, thoughtful treatment systems can be designed, particularly in industrial systems in which ammonia concentrations may be lower. Figure 4-3 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 C4), but genistein degradation was unaffected (Figure 4-3). 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 suspended solids, was observed during the approximately 50-hour experiment (data not shown).

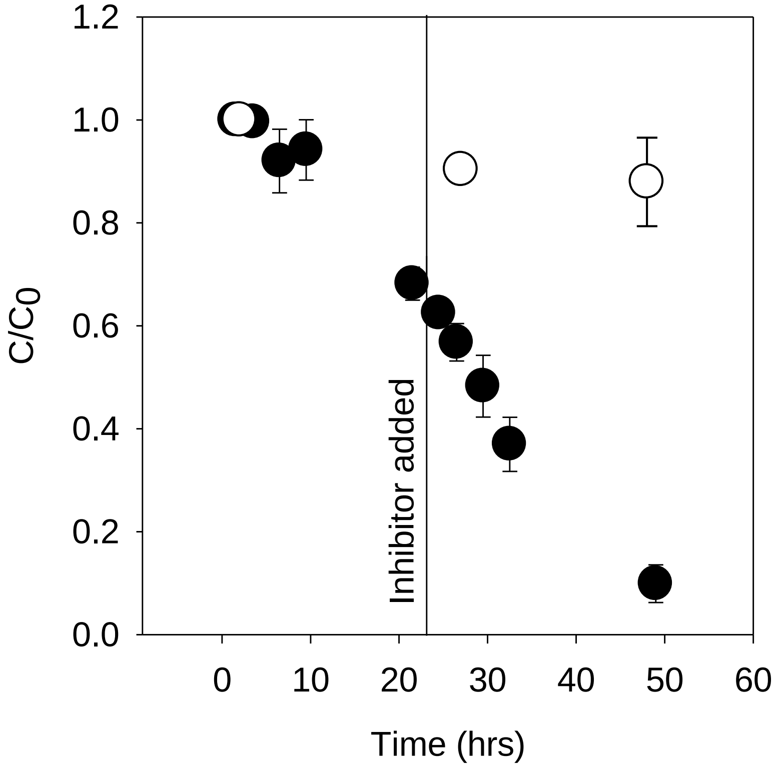


Figure 4-3. Degradation of genistein in an enriched nitrifying culture (black) and a killed control of the same culture (white) before and after the addition of allylthiourea, an inhibitor of ammonia oxidation.

Minnow Exposure to Phytoestrogen Degradates

The rapid attenuation of genistein and daidzein suggests that they pose little ecological risk to aquatic wildlife. Nevertheless, to truly assess ecological risk, one must consider whether the parent compounds' estrogenic activity is also rapidly attenuated. Upon exposure of fathead minnow larvae to microbiologically degraded phytoestrogens (genistein, daidzein, and formononetin, singly and in a mixture), no effects on survival, escape velocity, or total escape response were observed in comparison to an ethanol carrier control (Figures C5-6). Likewise, when adult minnows were exposed to the degradates of a mixture of genistein, daidzein, and formononetin, no statistically significant impacts on vitellogenin induction, secondary sex characteristics, histopathology, nest defense, or body condition were observed (Figures C7-14). 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 C15). This suggests that although the phytoestrogen degradates are not estrogenic and do not appear to impact larval survival, one or more products of degradation may have androgenic characteristics or may act as an estrogen antagonist during female egg production.

Environmental Significance

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 4-1 and 4-2). This degradation is apparently carried out 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.[15] A threshold limit of 1000 ng/L below which there is no effect on aquatic wildlife has been suggested, which is in agreement with recent observations.[15,37] Assuming zero-order kinetics and a rate of 8,820 ng genistein/(L×hr) (Table C6), an effluent containing 151,000 ng/L genistein would be reduced to 1000 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 periods degradation rates drop (Figure 4-2, Table C5-6) and these compounds could build-up in the water column or in sediment (Chapter 3) and impact aquatic wildlife as a result.[37]

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. Pulp and paper mill effluent, which has been shown to contain genistein, has also been shown to have similar androgenic effects on both mosquitofish and goldfish.[8,38,39] 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.

References

(1) Franke, A.; Custer, L.; Cerna, C. Quantitation of phytoestrogens in legumes by HPLC. *J. Agr. Food Chem.* **1994**. *42*, 1905–1913.

(2) Kuhnle, G.; Dell'Aquila, C.; Low, Y. Extraction and quantification of phytoestrogens in foods using automated solid-phase extraction and LC/MS/MS. *Anal. Chem.* **2007**. *23*, 9234-9.

(3) Liggins, J.; Mulligan, A.; Runswick, S. Daidzein and genistein content of cereals. *Eur. J. Clin. Nutr.* **2002**. *10*, 961-6.

(4) Liggins, J.; Bluck, L.; Runswick, S.; Atkinson, C. Daidzein and genistein content of fruits and nuts. *J. Nutr. Biochem.* **2000**. *11*, 326-31.

(5) Gutendorf, B.; Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **2001**. *166*, 79-89.

(6) Jefferson, W.; Padilla-Banks, E. Disruption of the developing female reproductive system by phytoestrogens: genistein as an example. *Mol. Nutr. Food Res.* **2007**. *51*, 832-844.

(7) Kiparissis, Y.; Balch, G.; Metcalfe, T. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka Oryzias latipes. *Environ. Health Persp.* **2003**. *111*, 1158-63.

(8) Kiparissis, Y.; Hughes, R.; Metcalfe, C. Identification of the isoflavonoid genistein in bleached kraft mill effluent. *Environ. Sci. Tech.* **2001**. *35*, 2423-7.

(9) Kuiper, G.; Lemmen, J.; Carlsson, B.; Corton, J. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **1998**. *139*, 4252-63.

(10) Latonnelle, K.; Fostier, A.; Le Menn, F. Binding affinities of hepatic nuclear estrogen receptors for phytoestrogens in rainbow trout (Oncorhynchus mykiss) and Siberian sturgeon (Acipenser baeri) *Gen. Comp. Endocr.* **2002**. *129*, 69-79.

(11) Clotfelter, E.; Rodriguez, A. Behavioral changes in fish exposed to phytoestrogens. *Environ. Poll.* **2006**. *144*, 833-9.

(12) MacLatchy, D.; Vanderkraak, G. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharm.* **1995**. *134*, 305-12.

(13) Pelissero, C.; Le Menn, F.; Kaushick, S. Estrogenic effect of dietary soya bean meal on vitellogenesis in cultured siberian sturgeon Acipenser baeri. *Gen. Comp. Endocr.* **1991**. *83*, 447-57.

(14) Tremblay, L.; Kraak, G. Comparison between the effects of the phytosterol β-Sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. *Environ. Toxicol. Chem.* **1999**. *18*, 329-336.

(15) Lundgren, M.; Novak, P. Quantification of phytoestrogens in industrial waste streams. *Environ. Toxicol. Chem.* **2009**. *28*, 2318-23.

(16) Mahmood-Khan, Z.; Hall, E. Occurrence and removal of plant sterols in pulp and paper mill effluents. *J. Environ. Eng. Sci.* **2003**. *2*, 17-26.

(17) Low, Y.; Taylor, J.; Grace, P.; Dowsett, M. Polymorphisms in the CYP19 gene may affect the positive correlations between serum and urine phytoestrogen metabolites and plasma androgen concentrations in men. *J. Nutr*. **2005**. *135*, 2680-2686.

(18) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**. *531*, 229-237.

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

(20) Kang, J.; Price, W. Occurrence of phytoestrogens in municipal wastewater and surface waters. *J. Environ. Monitor.* **2009**. *11*, 1477-83.

(21) Burnison, B.; Hartmann, A.; Lister, A. A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. *Environ. Toxicol. Chem.* **2003**. *22*, 2243-50.

(22) Hartmann, N.; Erbs, M.; Wettstein, F. Environmental exposure to estrogenic and other myco-and phytotoxins. *Chimia.* **2008**. *62*, 364-7.

(23) Hoerger, C.; Wettstein, F. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ. Sci. Technol.* **2009**. *43*, 6151-7.

(24) Muller, M.; Rabenoelina, F.; Balaguer, P. Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. *Environ. Toxicol. Chem.* **2008**. *27*, 1649-58.

(25) 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*, 2089-99.

(26) Kelly, M.; Arnold, W. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environ. Sci. Technol.* **2012**. *46*, 5396-403.

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

(28) Denny, J. S. *Guidelines for the culture of fathead minnows Pimephales promelas for the use in toxicity tests*. EPA/600/3-87.001. **1987**.

(29) McGee, M. R.; Julius, M. L.; Vajda, A. M.; Norris, D. O.; Barber, L. B.; Schoenfuss, H. L. Predator avoidance performance of larval fathead minnows (Pimephales promelas) following short-term exposure to estrogen mixtures. *Aquat. Toxicol.* **2009**, *91*, 355-361.

(30) Painter, M.; Buerkley, M.; Julius, M. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (Pimephales promelas). *Environ. Toxicol. Chem.* **2009**. *28*, 2677-84.

(31) Schultz, M.; Painter, M.; Bartell, S.; Logue, A. Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. *Aquat. Toxicol.* **2011**. *104*, 38-47.

(32) Fulton, T. W. The rate of growth of fishes. *Fisheries Board of Scotland, Annual Report* 22(3):141-241 **1904.**

(33) Wolf, J. C. Histology and histopathology guidelines for phase 1V of OECD fish screening assay for EDCs; WA #04-14; US Environmental Protection Agency EPL Project No. 481-017, Washington, DC. **2008**.

(34) Shappell, N.; Hyndman, K.; Bartell, S. Comparative biological effects and potency of 17α-and 17β-estradiol in fathead minnows. *Aquat. Toxicol.* **2010**. *100*, 1-8.

(35) Dammann, A.; Shappell, N.; Bartell, S. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, Pimephales promelas. *Aquat. Toxicol.* **2011**. *105*, 559-68.

(36) Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Res.* **2004**. *38*, 2322-9.

(37) Rearick, D. C.; Fleischhacker, N. T.; Kelly, M. M.; Arnold, W. A.; Novak, P. J.; Schoenfuss, H. L. Environmenal occurrence of phytoestrogens and exposure effects on fathead minnows. In preparation.

(38) Ellis, R.; van den Heuvel, M.; Bandelj, E. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**. *22*, 1448-56.

(39) Orlando, E.; Bass, D.; Caltabiano, L.; Davis, W.; Gray Jr., L.; Guillete Jr., L. Altered development and reproduction in mosquitofish exposed to pulp and paper mill effluent in the Fenholloway River, Florida USA. *Aquat. Toxicol.* **2007**, *84*, 399-405.

Chapter 5. Fate of Phytoestrogens in Model Aquatic Systems and Recommendations

With the understanding of the chemical properties and biodegradability of genistein and daidzein developed in Chapters 2-4, it is now possible to predict their environmental fate under specified environmental conditions. To do so, a box model representing a lake or pond, and a multi-box model representing a stream or river are developed (Figure 5-1). First, the site-specific parameters controlling genistein and daidzein fate are reviewed.

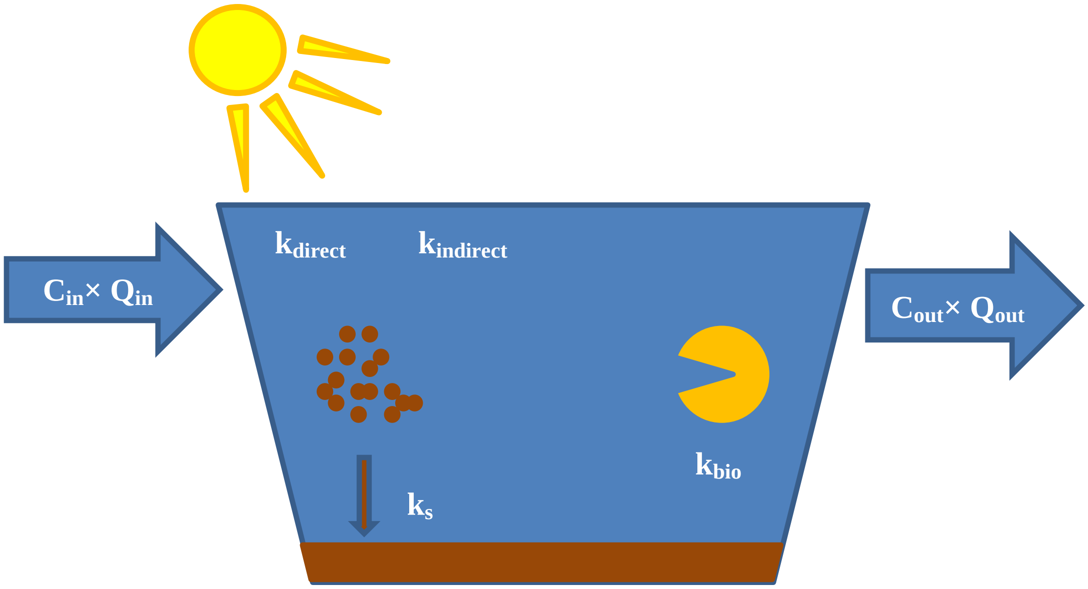


Figure 5-1. The concentration of genistein or daidzein in a lake will depend on the inputs, outputs, and in-lake attenuation processes including direct and indirect photolysis, biodegradation, and sorption to settling particles.

The rate constants for direct and indirect photolysis were developed in Chapter 2, the equilibria governing sorption to particles were measured in Chapter 3, and the rates of biodegradation were quantified in Chapter 4.

The rate of direct photolysis is given by the equation

$$\frac{d[C]}{dt} = -k_a\phi[C] \tag{5-1}$$

where k_a is the rate of light absorption by the molecule, ϕ is the quantum yield, and $[C]$ is the aqueous concentration of the molecule. The rate of light absorption is given by the overlap of the solar spectrum and the absorption spectrum of the molecule. The solar spectrum reaching the earth's surface changes magnitude with time of day and latitude. The spectrum also changes as a function of water depth, and with light screening in a body of water. For some molecules, including genistein and daidzein, the absorption spectrum is dependent on pH, as the pH of the surrounding water governs the degree of resonance in the molecule. Molecules with multiple protonation states may also have different quantum yields for each protonation state, as demonstrated for genistein and daidzein in Chapter 2.

The rates of indirect photolysis depend on the steady-state concentrations of the photochemically produced reactive intermediates (PPRIs) involved, according to the equation

$$\frac{dC}{dt} = -k_{PPRI}[C][PPRI] \tag{5-2}$$

where k_PPRI is the second-order rate constant and $[PPRI]$ is the steady-state concentration of the PPRI in question. Second-order rate constants are given in Chapter 2 for hydroxyl radical and singlet oxygen. Triplet-state natural organic matter was also shown to be important for the photodegradation of genistein, but a rate constant was not quantified due to the highly variable nature of NOM composition and ^{3}NOM* reactivity. The concentration of singlet oxygen is governed by the reaction of triplet oxygen with ^{3}NOM*. Typical singlet oxygen concentrations range from 10^{-14} to 10^{-12} M. The concentration of hydroxyl radical is more complex and less well-understood. It can be produced from ^{3}NOM*, NO_3^-, and reactions involving Fe^{2+}. Typical environmental concentrations range from 10^{-17} to 10^{-15} M.[1]

The equilibria governing the sorption of genistein and daidzein to particles were developed in Chapter 3. Sorption may represent a number of processes, demonstrated by the equation

$$f_s = 1 - 1/(1 + r_{sw}(f_{oc}K_{oc} + f_{kao}K_{kao} + f_{gt}K_{gt} + f_{NaM}K_{NaM}) \qquad (5\text{-}3)$$

where f_s is the fraction of a molecule sorbed to particles, r_{sw} is the solid-water ratio or suspended sediment concentration, f_x is the fraction of organic carbon, kaolinite, goethite, or Na-montmorillonite, and K_x is the distribution coefficient for the molecule sorbing to organic carbon, kaolinite, goethite, or Na-montmorillonite. Particles with sorbed phytoestrogens settle according to the sedimentation rate constant (k_s), given by the equation

$$k_s = \frac{v_s}{h} \qquad (5\text{-}4)$$

where v_s is the settling velocity of the particles, and h is the depth of the water body. Assuming a constant equilibrium between the sorbed and dissolved phases, a first-order equation can be written to describe the loss of genistein or daidzein with particles:

$$\frac{dC_{tot}}{dt} = -k_s f_s C_{tot} \qquad (5\text{-}5)$$

where C_{tot} represents the sum of genistein or daidzein in the sorbed and dissolved phases.

In Chapter 4, rates of biodegradation under different conditions were investigated. At high concentrations, the data were best fit to a zero-order model,

$$\frac{dC}{dt} = -k_{bio} \qquad (5\text{-}6)$$

where k_{bio} is the zero-order rate constant.

Taken together, these equations can be assembled into a simple box model describing the concentration of genistein or daidzein in a lake or pond over time:

$$\frac{dC_{tot}}{dt} = \frac{1}{V}(C_{tot,in}Q_{in} - C_{tot}Q_{out}) - k_a\phi(1 - f_s)[C_{tot}] - k_{PPRI}(1 - f_s)[C_{tot}][PPRI] - k_s f_s C_{tot} - k_{bio}(1 - f_s)) \qquad (5\text{-}7)$$

where V is the volume of the pond, C_{in} and C are the average concentrations of inputs and outputs, and Q_{in} and Q_{out} are the total water flux of the inputs and outputs. This is a first-order linear inhomogenous differential equation of the form

$$\frac{dC}{dt} = J(t) - k(t)C \qquad (5\text{-}8)$$

where J is the sum of terms dependent on time, but not concentration (here, $C_{in}Q_{in}$ and k_{bio}) and k is the sum of terms dependent on time and multiplied by concentration (here, $\mathrm{Q}_{\mathrm{out}}$, $k_a\phi$, $k_{PPRI}\,[PPRI]$, and $k_s f_s$). The solution to an equation of this form is

$$C(t) = J_0 e^{-kt} + \frac{J}{k}(1 - e^{-kt}) \qquad (5\text{-}9)$$

where $\frac{J}{k}$ represents the steady state concentration of C.[1]

A box model based on Greifensee, a well parameterized lake in Switzerland, was constructed using equation 5-9. Parameters informing the model are in Table 5-1. Results of the model are presented in Figures 5-2 through 5-5. Models were constructed assuming a pH of 8.5, in winter and in summer, for genistein and daidzein. Sorption is assumed to be constant, while biological degradation is assumed to slow to 25% of its summer rate in winter. Rates of direct photolysis are taken from Chapter 2. The concentrations of hydroxyl radical and singlet oxygen are assumed to be at the high end of the range presented in Schwarzenbach et al.[1] in the summer, and at the low end in the winter.

Table 5-1. Parameters used as inputs to the box model

	Summer, GEN	Winter, GEN	Summer, DDZ	Winter, DDZ
V (m^3)	1.50×10^8	1.50×10^8	1.50×10^8	1.50×10^8
C_{eff} (ng/L)	1.50×10^6	1.50×10^6	9.8×10^5	9.8×10^5
f_{eff}	0.1	0.1	0.1	0.1
Q (m^3/day)	3.40×10^5	3.40×10^5	3.40×10^5	3.40×10^5
k_{direct} (/hr)	1.95×10^{-2}	5.87×10^{-3}	2.04	0.63
k_{OH} (/hr)	8.71×10^{-6}	8.71×10^{-8}	6.94×10^{-6}	6.94×10^{-8}
k_{1O2}(/hr)	3.57×10^{-5}	3.57×10^{-7}	1.84×10^{-5}	1.84×10^{-7}
k_{NOM} (/hr)	6.93×10^{-2}	6.93×10^{-2}	6.30×10^{-1}	6.30×10^{-1}
k_s (/hr)	4.79×10^{-3}	4.79×10^{-3}	4.79×10^{-3}	4.79×10^{-3}
f_s	1.97×10^{-2}	1.97×10^{-2}	8.1×10^{-3}	8.1×10^{-3}
k_{bio} (µg/(L × hr))	8950	1430	6490	1622.5
C_0	0	0	0	0

The rate constant for the reaction with ^{3}NOM* is calculated based on the observed half-lives of genistein in daidzein in Mississippi River water in Chapter 2. The sedimentation rate is based on the settling velocity reported in Stoll et. al[2] and the depth reported in Schwarzenbach et al.[1] The volume (*V*) and flow (*Q*) are also found in Schwarzenbach et al.[1]. The effluent fraction (f_{eff}) is reported in Tixier et al.[3] The models shown describe the concentration of genistein or daidzein in the lake in a scenario where the treatment plant 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.[4] The steady-state concentration reached for each scenario is given in Table 5-2.

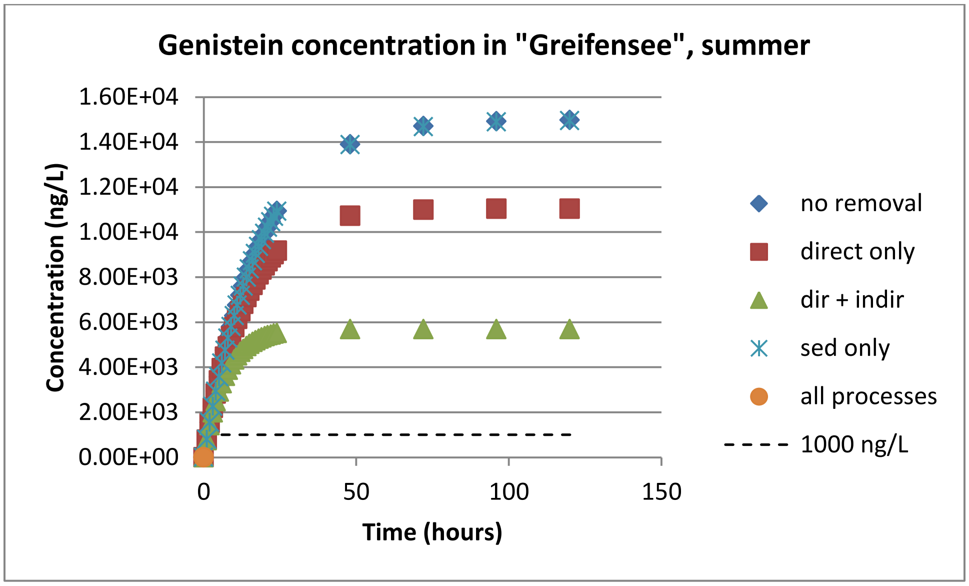


Figure 5-2. Genistein concentration in Greifensee in summer with no removal, removal by direct photolysis only, direct and indirect photolysis, sedimentation only, or direct and indirect photolysis, sedimentation, and biodegradation. Including the zero-order rate for biodegradation forces the concentration below 0 at the second time point. The threshold concentration of 1000 ng/L is shown for reference.

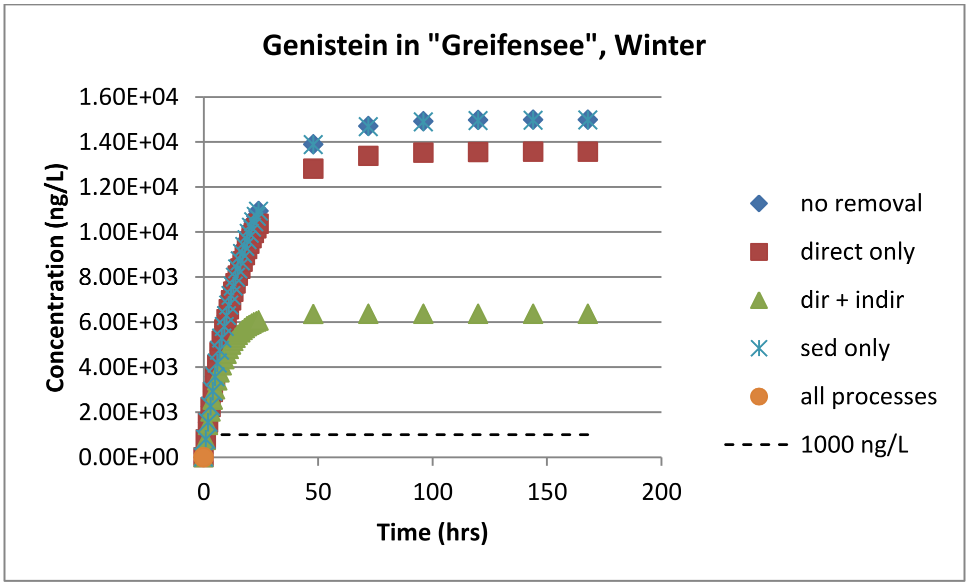


Figure 5-3. Genistein concentration in Greifensee in winter with no removal, removal by direct photolysis only, direct and indirect photolysis, sedimentation only, or direct and indirect photolysis, sedimentation, and biodegradation. Including the zero-order rate for biodegradation forces the concentration below 0 at the second time point. The threshold concentration of 1000 ng/L is shown for reference.

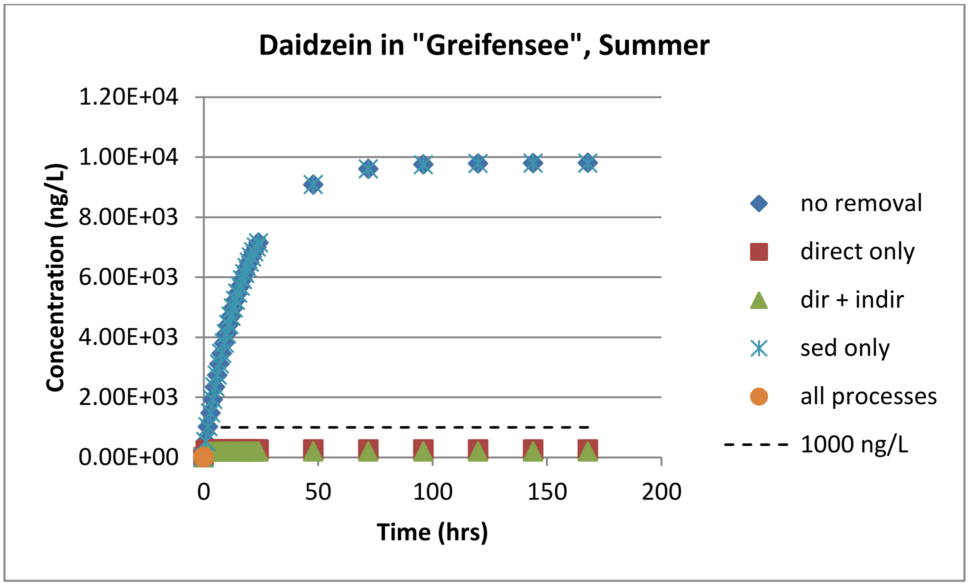


Figure 5-4. Daidzein concentration in Greifensee in summer with no removal, removal by direct photolysis only, direct and indirect photolysis, sedimentation only, or direct and indirect photolysis, sedimentation, and biodegradation. Including the zero-order rate for biodegradation forces the concentration below 0 at the second time point. The threshold concentration of 1000 ng/L is shown for reference.

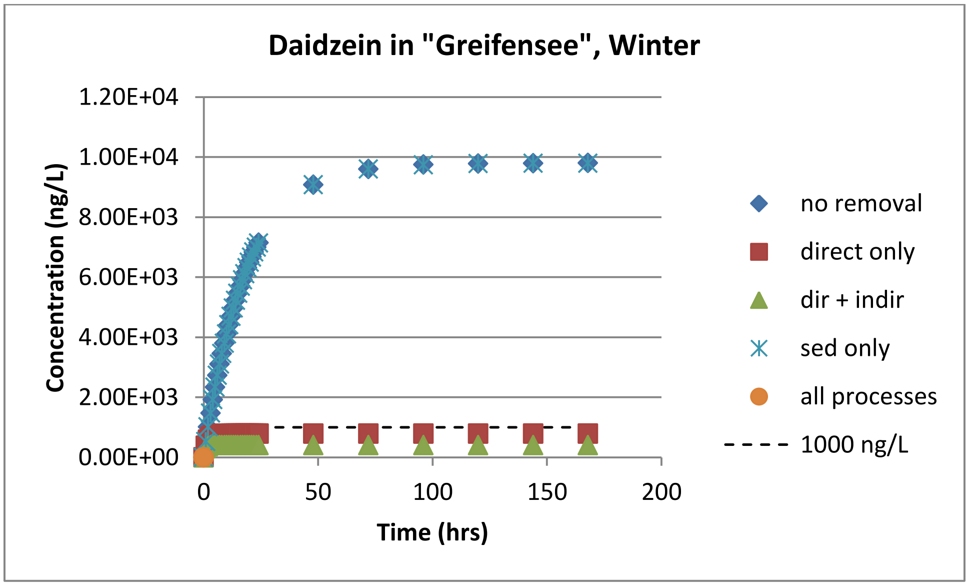


Figure 5-5. Daidzein concentration in Greifensee in summer with no removal, removal by direct photolysis only, direct and indirect photolysis, sedimentation only, or direct and indirect photolysis, sedimentation, and biodegradation. Including the zero-order rate for biodegradation forces the concentration below 0 at the second time point. The threshold concentration of 1000 ng/L is shown for reference.

Table 5-2. Steady-state concentrations for genistein and daidzein in summer and winter in Greifensee

	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
Direct only	1.11×10^4	1.36×10^4	2.60×10^2	7.93×10^2
Direct and Indirect	5.77×10^3	6.37×10^3	2.00×10^2	4.13×10^2
Bio only	-1.46×10^5	-2.53×10^4	-1.07×10^5	-1.94×10^4
Sedimentation only	1.50×10^4	1.50×10^4	9.78×10^2	9.78×10^2
All processes	-5.62×10^4	-4.57×10^3	-2.18×10^3	-8.20×10^2

To evaluate the persistence of genistein and daidzein in streams, a multi-box model was constructed. For box 0, equation 9 was used. For box n+1, equation 9 was amended such that its inflow concentration is equal to the outflow concentration of box n, rather than C_{eff}. Two streams were imagined, one ("Minnesota River") with a higher Q (20,000 m^3/hr) and lower f_{eff} (0.05), and one ("Zumbro River") with a lower Q (500 m^3/hr) and a higher f_{eff} (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. The results for daidzein are found in Figures 5-6 and 5-7. In-stream removal processes other than biodegradation for genistein appeared not to compete with dilution effects, so the results are not shown. Zero-order biodegradation did not allow any accumulation of genistein or daidzein. Direct photolysis of daidzein appears to speed the rate of loss very slightly.

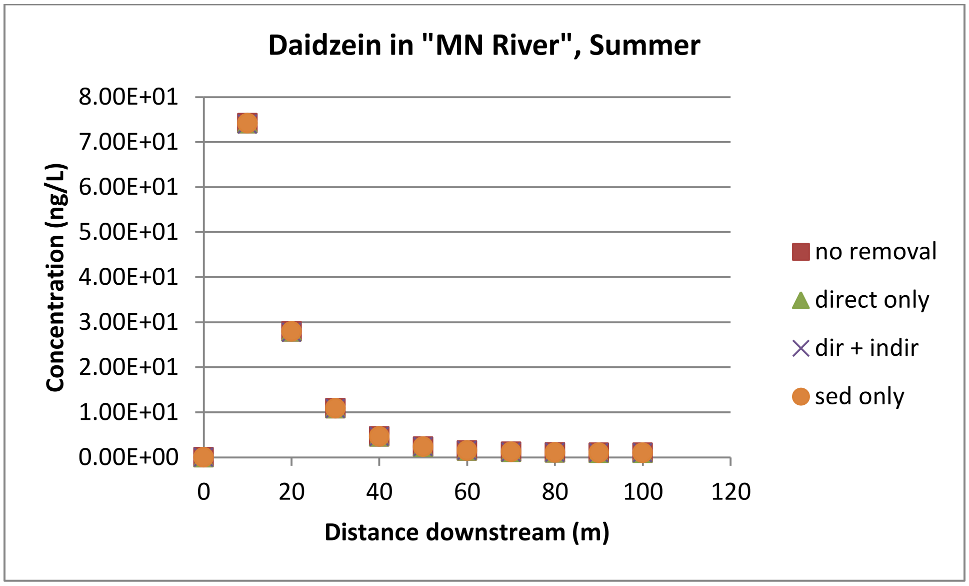


Figure 5-6. Daidzein concentration downstream of an effluent discharging into a fast-flowing river. Adding in the zero-order biodegradation term forces the concentration below zero immediately, so biodegradation is not shown.

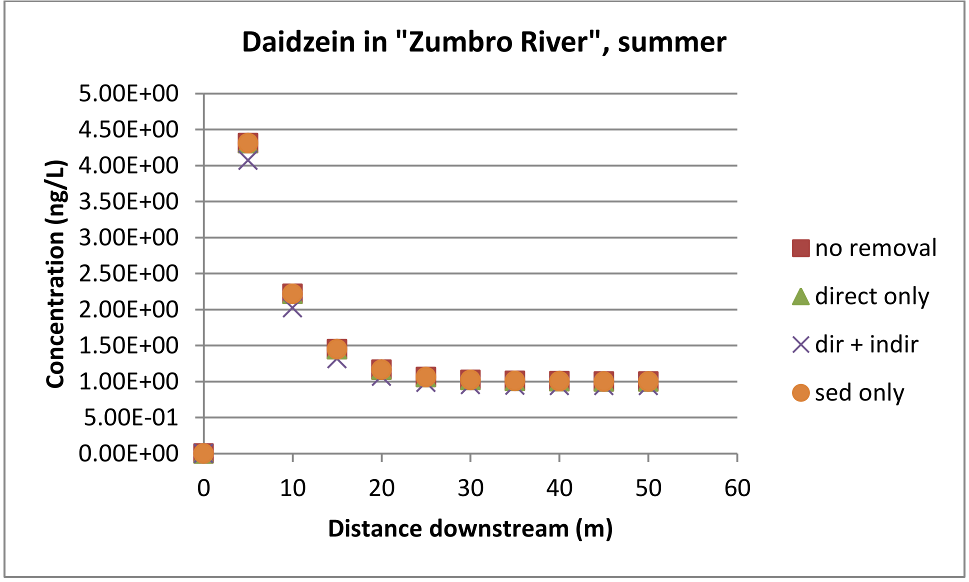


Figure 5-7. Daidzein concentration downstream of an effluent discharging into a slower-moving river. Adding in the zero-order biodegradation term forces the concentration below zero immediately, so biodegradation is not shown.

From these models we see quite clearly that where zero-order 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. Because the biodegradation products of genistein and daidzein have been shown to possess androgenic or anti-estrogenic activity, caution should still be exercised.

Future research could focus on identifying the products of photolysis and biodegradation, to better address concerns about ecological effects of phytoestrogen discharges. In addition to identifying the products of photolysis, it would be helpful to know their ecological effects. Finally, although sorption to particles appears to be a minor process in the removal of phytoestrogens, they have been detected in soils. Eroding soils could be a source of sorbed phytoestrogens to the aquatic environment. Thus, it would be interesting to look for phytoestrogens in sediments and associated porewaters to see if they accumulate in sediments over time, or if they are degraded by sediment microorganisms.

References

(1) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

(2) Stoll, J.; Ulrich, M.; Giger, W. Dynamic behavior of fluorescent whitening agents in Greifensee: Field measurements combined with mathematical modeling of sedimentation and photolysis. *Environ. Sci. Technol.* **1998**, *32*, 1875–1881.

(3) Tixier, C.; Singer, H. P.; Canonica, S.; Müller, S. R. Phototransformation of Triclosan in Surface Waters: A Relevant Elimination Process for This Widely Used BiocideLaboratory Studies, Field Measurements, and Modeling. *Environ. Sci. Technol.* **2002**, *36*, 3482–3489.

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

Bibliography

Chapter 1 References

(1) Lapcík, O. Isoflavonoids in non-leguminous taxa: a rarity or a rule? *Phytochemistry 68*, 2909–16.

(2) Thompson, L. U.; Boucher, B. A.; Liu, Z.; Cotterchio, M.; Kreiger, N. Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. *Nutr. Cancer* **2006**, *54*, 184–201.

(3) Liggins, J.; Bluck, L. J. C.; Runswick, S.; Atkinson, C.; Coward, W. A.; Bingham, S. A. Daidzein and genistein contents of vegetables. *Brit. J. Nutr.* **2000**, *84*, 717–725.

(4) De Sanctis, F.; Silvestrini, M. G.; Luneia, R.; Botondi, R.; Bellincontro, A.; Mencarelli, F. Postharvest dehydration of wine white grapes to increase genistein, daidzein and the main carotenoids. *Food Chem.* **2012**, *135*, 1619–25.

(5) Mazur, W.; Adlercreutz, H. Naturally occurring oestrogens in food. *Pure Appl. Chem.* **1998**, *70*, 1759–1776.

(6) National Biodiesel Board. Biodiesel Basics http://www.biodiesel.org/what-is-biodiesel/biodiesel-basics. Accessed 7-22-2013.

(7) Energy Information Administration U.S. Inputs to Biodiesel Production http://www.eia.gov/biofuels/biodiesel/production/table3.pdf. Accessed 7-22-2013.

(8) Biodiesel Industry Aims For 10% of On-Road Diesel Market by 2022 http://www.biofuelsjournal.com/articles/Biodiesel_Industry_Aims_For_10__of_On_Road_Diesel_Market_by_2022-130159.html. Accessed 7-22-2013.

(9) Chevron Corporation. Diesel Fuels Technical Review **2007**, 116 pp.

(10) International Road Federation, World Road Statistics, and I. E. A. Road sector diesel fuel consumption per capita http://data.worldbank.org/indicator/IS.ROD.DESL.PC. Accessed 7-22-2013.

(11) United Nations Population Division, United Nations Statistical Division, Eurostat: Demographic Statistics, and Secretariat of the Pacific Community, and U. S. C. B. I. D. Population, total http://data.worldbank.org/indicator/SP.POP.TOTL. Accessed 7-22-2013

(12) Committee on Water Implications of Biofuels Production in the United States, N. R. C. *Water Implications of Biofuels Production in the United States*; National Academies Press: Washington, D.C., 2008; p. 88.

(13) Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B.; Gustafsson, J. a Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **1998**, *139*, 4252–63.

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

(15) Ferrer, I.; Barber, L. B.; Thurman, E. M. Gas chromatographic-mass spectrometric fragmentation study of phytoestrogens as their trimethylsilyl derivatives: identification in soy milk and wastewater samples. *J. Chromatogr. A* **2009**, *1216*, 6024–32.

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

(17) Spengler, P.; Körner, W.; Metzger, J. W. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. *Environ. Toxicol. Chem.* **2001**, *20*, 2133–2141.

(18) Pawlowski, S.; Ternes, T.; Bonerz, M.; Kluczka, T.; van der Burg, B.; Nau, H.; Erdinger, L.; Braunbeck, T. Combined in situ and in vitro assessment of the estrogenic activity of sewage and surface water samples. *Toxicol. Sci.* **2003**, *75*, 57–65.

(19) 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.

(20) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P.; Samperi, R.; Laganà, A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**, *531*, 229–237.

(21) 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 Sp.* **2006**, *20*, 2411–2418.

(22) Biggers, J. D.; Curnow, D. H. Oestrogenic activity of subterranean clover. I. The oestregenic activity of genistein. *Biochem. J.* **1954**, *58*, 278–282.

(23) 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.

(24) Sivesind, E.; Seguin, P. Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *J. Agr. Food Chem.* **2005**, *53*, 6397–402.

(25) 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. Agr. Food Chem.* **2007**, *55*, 8339–8345.

(26) Hoerger, C. C.; Wettstein, F. E.; Bachmann, H. J.; Hungerbühler, K.; Bucheli, T. D. Occurrence and mass balance of isoflavones on an experimental grassland field. *Environ. Sci. Technol.* **2011**, *45*, 6752–60.

(27) Kuster, M.; Azevedo, D. A.; López de Alda, M. J.; Aquino Neto, F. R.; Barceló, D. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). *Environ. Int.* **2009**, *35*, 997–1003.

(28) Ribeiro, C.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. *Arch. Environ. Con. Tox.* **2009**, *56*, 1–11.

(29) Ribeiro, C.; Pardal, M. A.; Martinho, F.; Margalho, R.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Distribution of endocrine disruptors in the Mondego River estuary, Portugal. *Environ. Monit. Assess.* **2009**, *149*, 183–93.

(30) 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.

(31) 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.

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

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

(34) 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.

(35) MacKay, A. A.; Vasudevan, D. Polyfunctional ionogenic compound sorption: challenges and new approaches to advance predictive models. *Environ. Sci. Technol.* **2012**, *46*, 9209–23.

(36) Sposito, G. *The Chemistry of Soils*; Oxford University Press, USA, 2008; p. 329.

(37) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

(38) Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Res.* **2004**, *38*, 2323–2330.

(39) Vader, J.; Van Ginkel, C.; Sperling, F.; De Jong, J.; De Boer, W.; De Graaf, J.; Van Der Most, M.; Stokman, P. Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere* **2000**, *41*, 1239–1243.

(40) Roh, H.; Subramanya, N.; Zhao, F.; Yu, C.-P.; Sandt, J.; Chu, K.-H. Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria. *Chemosphere* **2009**, *77*, 1084–9.

(41) Kim, J. Y.; Ryu, K.; Kim, E. J.; Choe, W. S.; Cha, G. C.; Yoo, I.-K. Degradation of bisphenol A and nonylphenol by nitrifying activated sludge. *Process Biochem.* **2007**, *42*, 1470–1474.

Chapter 2 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 Persp.* **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. Heal. A* **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 Persp.* **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. Agr. 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 Sp.* **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. J.; McNeill, K.; Arnold, W. A. 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 Jr, F. E.; 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

(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, N.J., 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.

Chapter 3 References

(1) 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 Persp.* **2003**, *111*, 1158–1163.

(2) 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.

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

(4) 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. Agr. Food Chem.* **2007**, *55*, 8339–8345.

(5) Hoerger, C. C.; Wettstein, F. E.; Bachmann, H. J.; Hungerbühler, K.; Bucheli, T. D. Occurrence and mass balance of isoflavones on an experimental grassland field. *Environ. Sci. Technol.* **2011**, *45*, 6752–60.

(6) 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.

(7) Hoerger, C. C.; Schenzel, J.; Strobel, B. W.; Bucheli, T. D. Analysis of selected phytotoxins and mycotoxins in environmental samples. *Anal. Bioanal. Chem.* **2009**, *395*, 1261–89.

(8) 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.

(9) Ribeiro, C.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. *Arch. Environ. Con. Tox.* **2009**, *56*, 1–11.

(10) Ribeiro, C.; Pardal, M. A.; Tiritan, M. E.; Rocha, E.; Margalho, R. M.; Rocha, M. J. Spatial distribution and quantification of endocrine-disrupting chemicals in Sado River estuary, Portugal. *Environ. Monit. Assess.* **2009**, *159*, 415–27.

(11) 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.

(12) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P.; Samperi, R.; Laganà, A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**, *531*, 229–237.

(13) 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 Sp.* **2006**, *20*, 2411–2418.

(14) Felcyn, J. R.; Davis, J. C. C.; Tran, L. H.; Berude, J. C.; Latch, D. E. Aquatic photochemistry of isoflavone phytoestrogens: Degradation kinetics and pathways. *Environ. Sci. Technol.* **2012**. *46*, 6698-6704.

(15) Kelly, M.; Arnold, W. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environ. Sci. Technol.* **2012**, *46*, 5396–5403.

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

(17) Stancanelli, R.; Mazzaglia, A.; Tommasini, S.; Calabrò, M. L.; Villari, V.; Guardo, M.; Ficarra, P.; Ficarra, R. The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: a spectroscopic investigation with implications in the pharmaceutical analysis. *J. Pharmaceut. Med.* **2007**, *44*, 980–4.

(18) Han, H.-K.; Lee, B.-J.; Lee, H.-K. Enhanced dissolution and bioavailability of biochanin A via the preparation of solid dispersion: in vitro and in vivo evaluation. *Int. J. Pharm.* **2011**, *415*, 89–94.

(19) Schenzel, J.; Goss, K.-U.; Schwarzenbach, R. P.; Bucheli, T. D.; Droge, S. T. J. Experimentally determined soil organic matter-water sorption coefficients for

different classes of natural toxins and comparison with estimated numbers. *Environ. Sci. Technol.* **2012**, *46*, 6118–6126.

(20) Rothwell, J.; Day, A. Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. *J. Agr. Food Chem.* **2005**, *53*, 4355–4360.

(21) US EPA Estimation Programs Interface Suite(TM) for Microsoft(R) Windows, v 4.1 **2013**.

(22) Sposito, G. *The Chemistry of Soils*; Oxford University Press, USA, 2008; p. 329.

(23) Shareef, A.; Angove, M. J.; Wells, J. D.; Johnson, B. B. Sorption of bisphenol A, 17alpha-ethynylestradiol and estrone to mineral surfaces. *J. Colloid Interf. Sci.* **2006**, *297*, 62–9.

(24) Van Emmerik, T.; Angove, M. J.; Johnson, B. B.; Wells, J. D.; Fernandes, M. B. Sorption of 17 [beta]-estradiol onto selected soil minerals. *J. Colloid Interf. Sci.* **2003**, *266*, 33–39.

(25) Figueroa, R. A.; Leonard, A.; MacKay, A. A. Modeling Tetracycline Antibiotic Sorption to Clays. *Environ. Sci. Technol.* **2004**, *38*, 476–483.

(26) Figueroa, R. A.; MacKay, A. A. Sorption of Oxytetracycline to Iron Oxides and Iron Oxide-Rich Soils. *Environ. Sci. Technol.* **2005**, *39*, 6664–6671.

(27) Doucette, W. Soil and sediment sorption coefficients. *Handbook of Property Estimation Methods for Chemicals: Environmental and Health Sciences* **2000**.

(28) Meylan, W.; Howard, P. H.; Boethling, R. S. Molecular topology/fragment contribution method for predicting soil sorption coefficients. *Environ. Sci. Technol.* **1992**, *26*, 1560–1567.

(29) Clotfelter, E. D.; McNitt, M. M.; Carpenter, R. E.; Summers, C. H. Modulation of monoamine neurotransmitters in fighting fish Betta splendens exposed to waterborne phytoestrogens. *Fish Physiol. Biochem.* **2010**, *36*, 933–43.

(30) Anschutz, A.; Penn, R. L. Reduction of crystalline iron(III) oxyhydroxides using hydroquinone: Influence of phase and particle size. *Geochem. T.* **2005**, *6*, 60-66.

(31) Overgaard, C. G. Adsorption of the phytoestrogens, genistein and daidzein, onto the iron oxide-hydroxide goethite **2012**. University of Stavanger.

(32) Wang, S.; Arnold, W. A. Abiotic reduction of dinitroaniline herbicides. *Water Res.* **2003**, *37*, 4191–201.

(33) Lyman, W.; Reehl, W.; Rosenblatt, D. *Handbook of chemical property estimation methods;* American Chemical Society: Washington, D.C., **1990**.

(34) Dowling, S.; Regan, F.; Hughes, H. The characterisation of structural and antioxidant properties of isoflavone metal chelates. *Journal of inorganic biochemistry* **2010**, *104*, 1091–8.

(35) Deng, H.; Van Berkel, G. J. Electrospray mass spectrometry and UV/visible spectrophotometry studies of aluminum(III)-flavonoid complexes. *J. Mass Spectrom.* **1998**, *33*, 1080–1087.

(36) Deng, Y.; Liu, L.; Luisa Barrientos Velázquez, A.; Dixon, J. B. The Determinative Role of the Exchange Cation and Layer-Charge Density of Smectite on Aflatoxin Adsorption. *Clay Clay Min.* **2012**, *60*, 374–386.

(37) Higgins, C. P.; Luthy, R. G. Sorption of Perfluorinated Surfactants on Sediments †. *Environ. Sci. Technol.* **2006**, *40*, 7251–7256.

(38) Dontsova, K. M.; Bigham, J. M. Anionic Polysaccharide Sorption by Clay Minerals. *Soil Sci. Soc. Am. J.* **2005**, *69*, 1026-1035.

(39) MacKay, A. A.; Canterbury, B. Oxytetracycline sorption to organic matter by metal-bridging. *J. Environ. Qual.* **2005**, *34*, 1964–71.

(40) Lützenkirchen, J. Ionic Strength Effects on Cation Sorption to Oxides: Macroscopic Observations and Their Significance in Microscopic Interpretation. *J. Colloid Interf. Sci.* **1997**, *195*, 149–155.

(41) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

Chapter 4 References

(1) Franke, A.; Custer, L.; Cerna, C. Quantitation of phytoestrogens in legumes by HPLC. *J. Agr. Food Chem.* **1994**. *42*, 1905–1913.

(2) Kuhnle, G.; Dell'Aquila, C.; Low, Y. Extraction and quantification of phytoestrogens in foods using automated solid-phase extraction and LC/MS/MS. *Anal. Chem.* **2007**. *23*, 9234-9.

(3) Liggins, J.; Mulligan, A.; Runswick, S. Daidzein and genistein content of cereals. *Eur. J. Clin. Nutr.* **2002**. *10*, 961-6.

(4) Liggins, J.; Bluck, L.; Runswick, S.; Atkinson, C. Daidzein and genistein content of fruits and nuts. *J. Nutr. Biochem.* **2000**. *11*, 326-31.

(5) Gutendorf, B.; Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **2001**. *166*, 79-89.

(6) Jefferson, W.; Padilla-Banks, E. Disruption of the developing female reproductive system by phytoestrogens: genistein as an example. *Mol. Nutr. Food Res.* **2007**. *51*, 832-844.

(7) Kiparissis, Y.; Balch, G.; Metcalfe, T. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka Oryzias latipes. *Environ. Health Persp.* **2003**. *111*, 1158-63.

(8) Kiparissis, Y.; Hughes, R.; Metcalfe, C. Identification of the isoflavonoid genistein in bleached kraft mill effluent. *Environ. Sci. Tech.* **2001**. *35*, 2423-7.

(9) Kuiper, G.; Lemmen, J.; Carlsson, B.; Corton, J. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **1998**. *139*, 4252-63.

(10) Latonnelle, K.; Fostier, A.; Le Menn, F. Binding affinities of hepatic nuclear estrogen receptors for phytoestrogens in rainbow trout (Oncorhynchus mykiss) and Siberian sturgeon (Acipenser baeri) *Gen. Comp. Endocr*. **2002**. *129*, 69-79.

(11) Clotfelter, E.; Rodriguez, A. Behavioral changes in fish exposed to phytoestrogens. *Environ. Poll.* **2006**. *144*, 833-9.

(12) MacLatchy, D.; Vanderkraak, G. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharm.* **1995**. *134*, 305-12.

(13) Pelissero, C.; Le Menn, F.; Kaushick, S. Estrogenic effect of dietary soya bean meal on vitellogenesis in cultured siberian sturgeon Acipenser baeri. *Gen. Comp. Endocr.* **1991**. *83*, 447-57.

(14) Tremblay, L.; Kraak, G. Comparison between the effects of the phytosterol β-Sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. *Environ. Toxicol. Chem.* **1999**. *18*, 329-336.

(15) Lundgren, M.; Novak, P. Quantification of phytoestrogens in industrial waste streams. *Environ. Toxicol. Chem.* **2009**. *28*, 2318-23.

(16) Mahmood-Khan, Z.; Hall, E. Occurrence and removal of plant sterols in pulp and paper mill effluents. *J. Environ. Eng. Sci.* **2003**. *2*, 17-26.

(17) Low, Y.; Taylor, J.; Grace, P.; Dowsett, M. Polymorphisms in the CYP19 gene may affect the positive correlations between serum and urine phytoestrogen metabolites and plasma androgen concentrations in men. *J. Nutr*. **2005**. *135*, 2680-2686.

(18) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**. *531*, 229-237.

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

(20) Kang, J.; Price, W. Occurrence of phytoestrogens in municipal wastewater and surface waters. *J. Environ. Monitor.* **2009**. *11*, 1477-83.

(21) Burnison, B.; Hartmann, A.; Lister, A. A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. *Environ. Toxicol. Chem.* **2003**. *22*, 2243-50.

(22) Hartmann, N.; Erbs, M.; Wettstein, F. Environmental exposure to estrogenic and other myco-and phytotoxins. *Chimia.* **2008**. *62*, 364-7.

(23) Hoerger, C.; Wettstein, F. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ. Sci. Technol.* **2009**. *43*, 6151-7.

(24) Muller, M.; Rabenoelina, F.; Balaguer, P. Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. *Environ. Toxicol. Chem.* **2008**. *27*, 1649-58.

(25) 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*, 2089-99.

(26) Kelly, M.; Arnold, W. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environ. Sci. Technol.* **2012**. *46*, 5396-403.

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

(28) Denny, J. S. *Guidelines for the culture of fathead minnows Pimephales promelas for the use in toxicity tests*. EPA/600/3-87.001. **1987**.

(29) McGee, M. R.; Julius, M. L.; Vajda, A. M.; Norris, D. O.; Barber, L. B.; Schoenfuss, H. L. Predator avoidance performance of larval fathead minnows (Pimephales promelas) following short-term exposure to estrogen mixtures. *Aquat. Toxicol.* **2009**, *91*, 355-361.

(30) Painter, M.; Buerkley, M.; Julius, M. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (Pimephales promelas). *Environ. Toxicol. Chem.* **2009**. *28*, 2677-84.

(31) Schultz, M.; Painter, M.; Bartell, S.; Logue, A. Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. *Aquat. Toxicol.* **2011**. *104*, 38-47.

(32) Fulton, T. W. The rate of growth of fishes. *Fisheries Board of Scotland, Annual Report* 22(3):141-241 **1904.**

(33) Wolf, J. C. Histology and histopathology guidelines for phase 1V of OECD fish screening assay for EDCs; WA #04-14; US Environmental Protection Agency EPL Project No. 481-017, Washington, DC. **2008**.

(34) Shappell, N.; Hyndman, K.; Bartell, S. Comparative biological effects and potency of 17α-and 17β-estradiol in fathead minnows. *Aquat. Toxicol.* **2010**. *100*, 1-8.

(35) Dammann, A.; Shappell, N.; Bartell, S. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, Pimephales promelas. *Aquat. Toxicol.* **2011**. *105*, 559-68.

(36) Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Res.* **2004**. *38*, 2322-9.

(37) Rearick, D. C.; Fleischhacker, N. T.; Kelly, M. M.; Arnold, W. A.; Novak, P. J.; Schoenfuss, H. L. Environmenal occurrence of phytoestrogens and exposure effects on fathead minnows. In preparation.

(38) Ellis, R.; van den Heuvel, M.; Bandelj, E. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**. *22*, 1448-56.

(39) Orlando, E.; Bass, D.; Caltabiano, L.; Davis, W.; Gray Jr., L.; Guillete Jr., L. Altered development and reproduction in mosquitofish exposed to pulp and paper mill effluent in the Fenholloway River, Florida USA. *Aquat. Toxicol.* **2007**, *84*, 399-405.

Chapter 5 References

(1) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2002.

(2) Stoll, J.; Ulrich, M.; Giger, W. Dynamic behavior of fluorescent whitening agents in Greifensee: Field measurements combined with mathematical modeling of sedimentation and photolysis. *Environ. Sci. Technol.* **1998**, *32*, 1875–1881.

(3) Tixier, C.; Singer, H. P.; Canonica, S.; Müller, S. R. Phototransformation of Triclosan in Surface Waters: A Relevant Elimination Process for This Widely Used BiocideLaboratory Studies, Field Measurements, and Modeling. *Environ. Sci. Technol.* **2002**, *36*, 3482–3489.

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

Appendix A References

(1) Ervens, B.; Gligorovski, S.; Herrmann, H. Temperature-dependent rate constants for hydroxyl radical reactions with organic compounds in aqueous solutions. *Phys. Chem. Chem. Phys.* **2003**, *5*, 1811-1824.

(2) 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.

(3) Mashiko, S.; Suzuki, N.; Koga, S.; Nakano, M.; Goto, T.; Ashino, T.; Mizumoto, I.; Inaba, H. Measurement of rate constants for quenching singlet oxygen with a Cypridina luciferin analog (2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-a]pyrazin-3-one) and sodium azide. *J. of Biolumin. Chemilumin.***1991**, *6*, 69-72.

(4) 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.

(5) 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.

Appendix A. Supporting Information for Chapter 2

Chemicals

Genistein (96%) and daidzein (95%) were purchased from TCI America. Acetic acid (100%), ammonium acetate (97%), sodium hydroxide (98.6%), sodium acetate (97.6%), potassium chloride (99.4%), disodium hydrogen phosphate (99.2%), phosphoric acid (85.6%), isopropyl alcohol (99.5%, hydroxyl radical quencher, k_{OH}=2.1 × 10^9 M^{-1} s^{-1})[1], hydrogen peroxide (29-32%), and sulfuric acid (96.4%) were purchased from Mallinckrodt. Acetonitrile (100%) and potassium dihydrogen phosphate (99.7%) were purchased from J.T. Baker. Hydrochloric acid (37%), methanol (99.9%), Rose Bengal (93%), and furfuryl alcohol (98%) were purchased from Sigma-Aldrich. Sodium tetraborate decahydrate (99.5%), 4-nitroacetophenone (PNAP, 97%), 4-nitroanisole (PNA, 99%), pyridine (99%) and acetophenone (99%) were purchased from Acros organics. Sorbic acid (99%, triplet quencher)[2] was purchased from Alfa Aesar. Sodium azide (99%, singlet oxygen quencher, $k_{^1O_2}$= 3.83 × 10^8 M^{-1} s^{-1})[3] was purchased from Janssen Chimica. Nitrogen (UHP/Zero-grade) and argon (99%) were purchased from Minneapolis Oxygen. All solvents were high pressure liquid chromatography (HPLC) grade and all reagents were ACS grade. Deionized (DI) water was obtained from a Milli-Q system.

Mississippi River water (MRW) was collected near the University of Minnesota on July 22, 2009 (NO_3^- =0.41 mg/L as N). It was filtered through a 0.7 μm glass fiber filter (Whatman GF/F) and adjusted to pH 12 using NaOH. Genistein (1.1 mg) and daidzein (1.7 mg) were added to separate 1 L volumes of the pH-adjusted MRW.

Similarly, 1 mg genistein and 1.5 mg daidzein were added to 1 L volumes of pH 12 DI water. This gave concentrations of 4.1 μM genistein in MRW, 3.7 μM genistein in DI water, 6.7 μM daidzein in MRW and 5.9 μM daidzein in DI water. A fraction of each solution was then refrigerated, while the remaining solution was separated into fractions, which were adjusted to pH 11, 8.7 or pH 5 using HCl and then refrigerated. A control experiment over a five-day period showed no degradation of genistein or daidzein prepared in this fashion.

HPLC Methods

All HPLC analyses were carried out using an Agilent 1200 series HPLC system with photodiode array detection and a Supelco Ascentis column (15 cm × 4.6 mm, 5μm particle diameter). When genistein or daidzein were analyzed alone, the mobile phase was 60% acetonitrile and 40% pH 5 acetate buffer containing 10% acetonitrile with a flow rate of 1 mL min^{-1}. Genistein was detected at 259 nm and daidzein was detected at 249 nm. Daidzein and acetophenone co-eluted using this method. To detect daidzein and acetophenone simultaneously, the mobile phase was 40% acetonitrile and 60% pH 5 acetate buffer.

Spectral Deconvolution

The UV-Vis spectrum of each protonation state of genistein and daidzein (Fig. 1, panels B and E) was determined using equation 5, which is a matrix equation of the form **Aa=b.**[4,5]

$$\begin{array}{l} pH\ 13.5 \\ \\ \\ pH\ 4.5 \end{array} \begin{pmatrix} \chi_{H_2DDZ} & \chi_{HDDZ^-} & \chi_{DDZ^{2-}} \\ \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots \end{pmatrix} \begin{pmatrix} \epsilon_{H_2DDZ,\lambda=190} & \cdots & \epsilon_{H_2DDZ,\lambda=400} \\ \epsilon_{HDDZ^-\ \lambda=190} & \cdots & \epsilon_{HDDZ^-\ \lambda=400} \\ \epsilon_{DDZ^{2-}\ \lambda=190} & \cdots & \epsilon_{DDZ^{2-}\ \lambda=400} \end{pmatrix} = \begin{array}{l} pH\ 13.5 \\ \\ \\ pH\ 4.5 \end{array} \begin{pmatrix} \epsilon_{tot,\lambda=190} & \cdots & \epsilon_{tot,\lambda=400} \\ \vdots & \ddots & \vdots \\ \vdots & \cdots & \vdots \end{pmatrix} \tag{S1}$$

The speciation matrix is represented by **A**, where χ is the molar fraction of each protonation state (for daidzein H_2DDZ, $HDDZ^-$, or DDZ^{2-} and for genistein H_3GEN, H_2GEN^-, $HGEN^{2-}$, GEN^{3-}) at a given pH from 4.5-13.5. The component spectra are represented by **a**, where ε is the molar absorptivity of a particular protonation state at a particular wavelength λ. The observed spectra are represented by **b**, where ε_{tot} represents the molar absorptivity of genistein or daidzein at each pH. Equation S1 is for daidzein, and the equation for genistein contains additional columns and rows for the additional protonation state. First, a speciation matrix was generated using the pK_a values. Then, the component spectra (**a**) were solved by using the left inverse of the speciation matrix, $\mathbf{(A^TA)^{-1}*A^T*b.}$ [4]

The data shown in figures S2 and S3 were analyzed by linear regression as described in the main text.

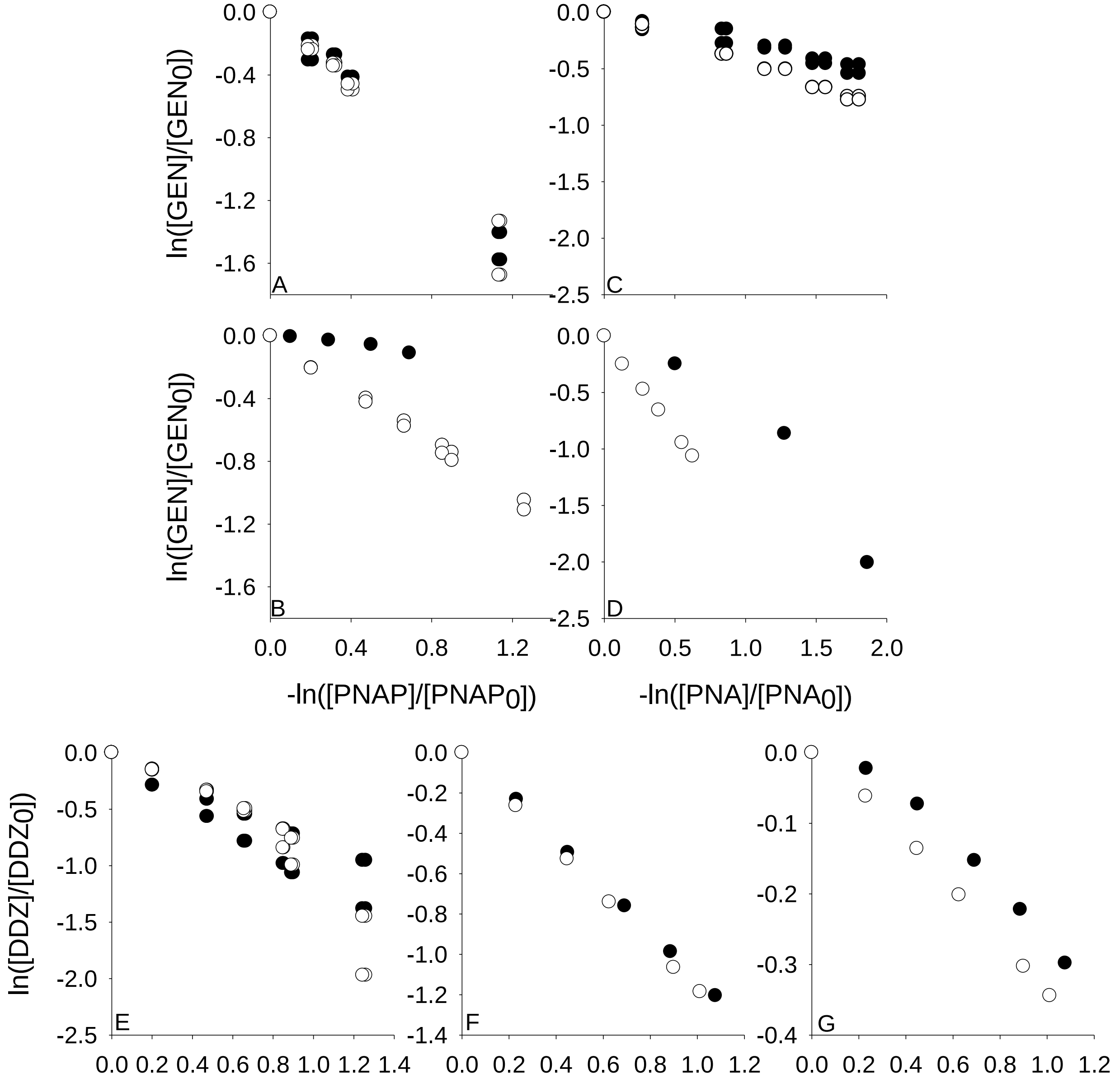


Figure A-1. Loss of genistein at pH 5 (A) , 8.7 (B), 11 (C), or 12 (D) and daidzein at pH 5 (E), 8.7 (F), and 12 (G) in DI water (filled) or MRW (outlined) versus loss of PNAP or PNA.

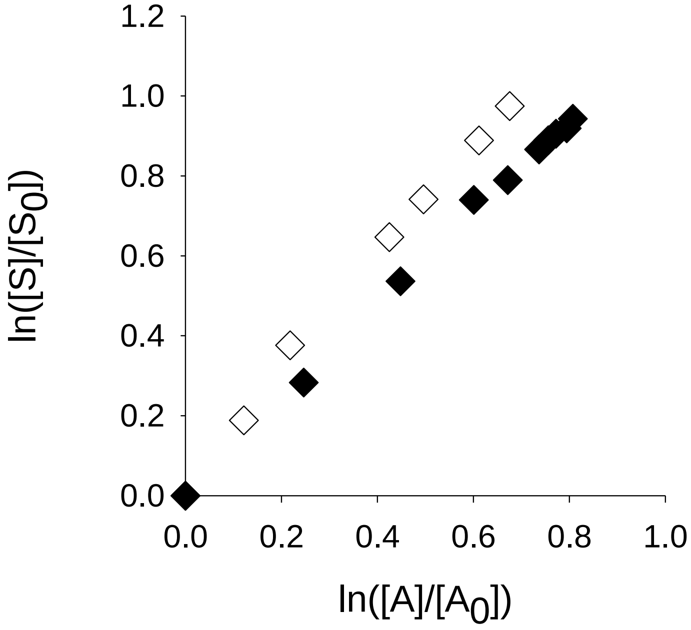


Figure A-2. Loss of genistein (outlined) or daidzein (filled) versus loss of acetophenone in the presence of Fenton’s reagent.

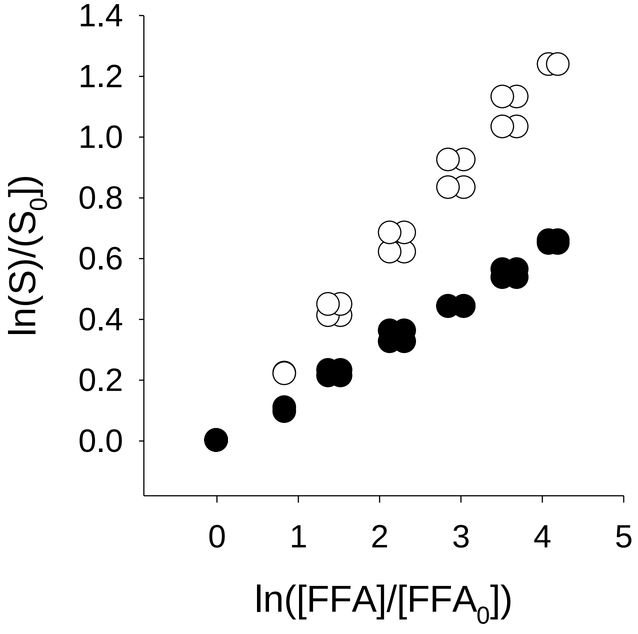


Figure A-3. Loss of genistein (outlined) or daidzein (filled) versus loss of furfuryl alcohol in the presence of Rose Bengal and simulated sunlight.

References

(1) Ervens, B.; Gligorovski, S.; Herrmann, H. Temperature-dependent rate constants for hydroxyl radical reactions with organic compounds in aqueous solutions. *Phys. Chem. Chem. Phys.* **2003**, *5*, 1811-1824.

(2) 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.

(3) Mashiko, S.; Suzuki, N.; Koga, S.; Nakano, M.; Goto, T.; Ashino, T.; Mizumoto, I.; Inaba, H. Measurement of rate constants for quenching singlet oxygen with a Cypridina luciferin analog (2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-a]pyrazin-3-one) and sodium azide. *J. of Biolumin. Chemilumin.***1991**, *6*, 69-72.

(4) 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.

(5) 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.

Appendix B. Supporting information for Chapter 3

Titration details

Kaolinite was titrated as described in the methods to estimate the pK_a of its amphoteric surface groups. The resulting titration curve is found in figure S6. The line of best fit (eq. B-1) was calculated using Microsoft Excel 2010.

$$pH = -68.6\,Q + 3.45 \tag{B-1}$$

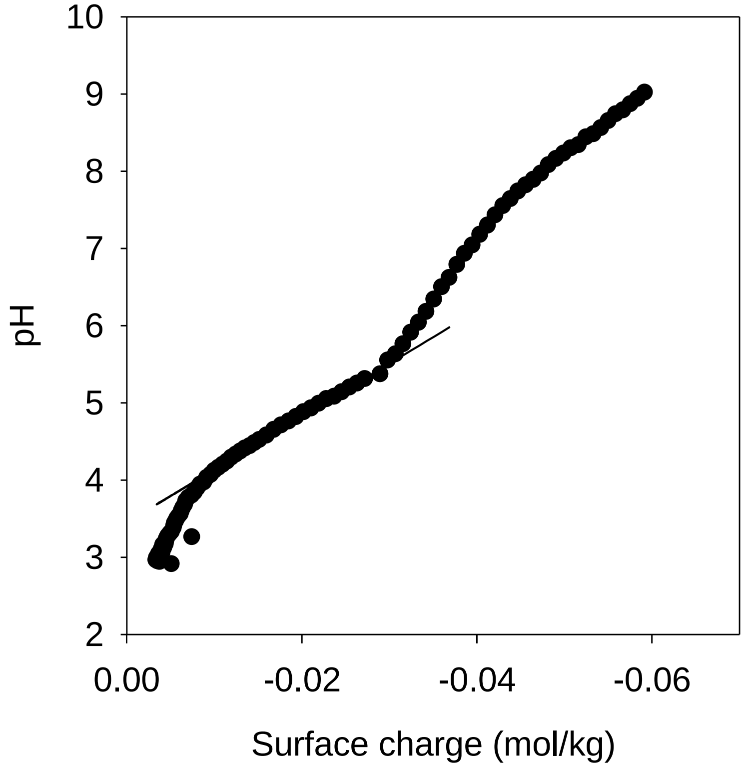


Figure B-1. Titration of a 5% kaolinite suspension in 10 mM KCl, by 0.78 M KOH.

Sorption Isotherms

Sorption isotherms for genistein, daidzein, and formononetin on goethite are shown in Figure S1. Daidzein did not sorb appreciably at pH 8.2 so the isotherm is omitted. Formononetin was not evaluated at pH 5.5, 6.2, or 7.1

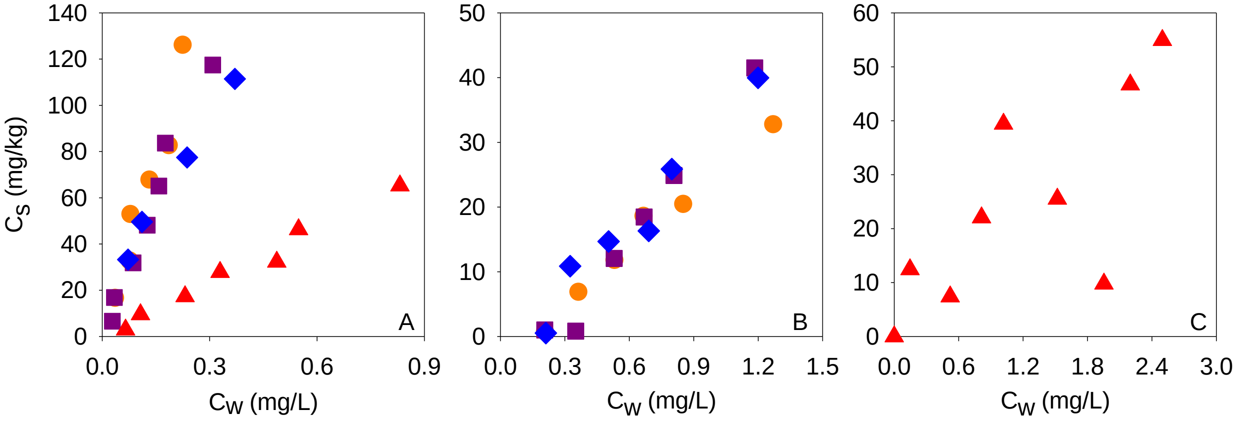


Figure B-2. Genistein (A), daidzein (B), and formononetin (C) isotherms on goethite.Yellow circles represent pH 5.5, purple squares represent pH 6.2, blue diamonds represent pH 7.1, and red triangles represent pH 8.2.

Isotherms for genistein on clay minerals are shown in Figure S2. All isotherms except Ca-montmorillonite at pH 11 are linear.

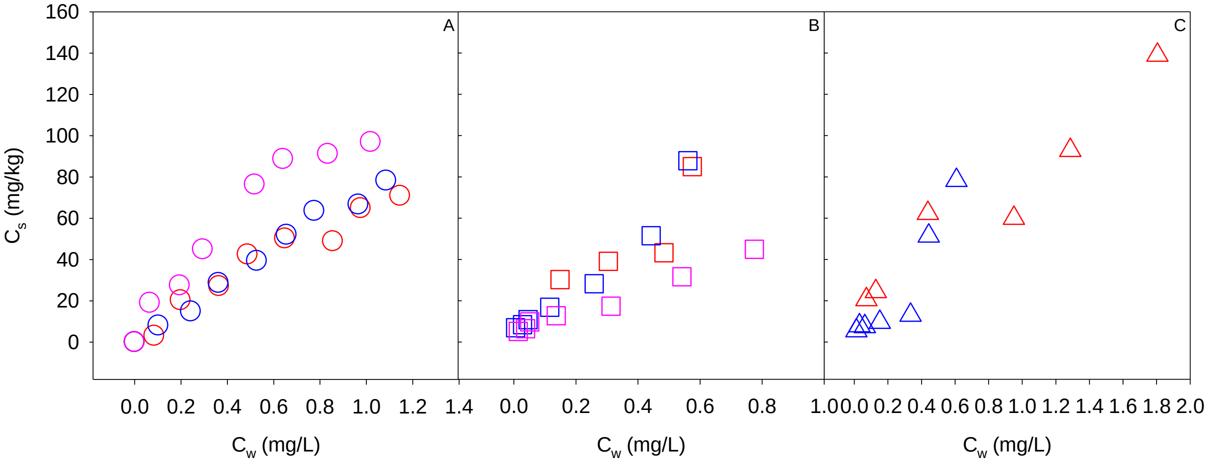


Figure B-3. Genistein sorption to Ca-montmorillonite (A), Na-montmorillonite (B), and kaolinite (C) at pH 6 (blue) pH 8.5 (red), and pH 11 (pink).

Isotherms for biochanin A on clay minerals at pH 8.5 are shown in Figure S3. All three isotherms are linear. Biochanin A sorbs much less strongly to Na-montmorillonite than Ca-montmorillonite or kaolinite.

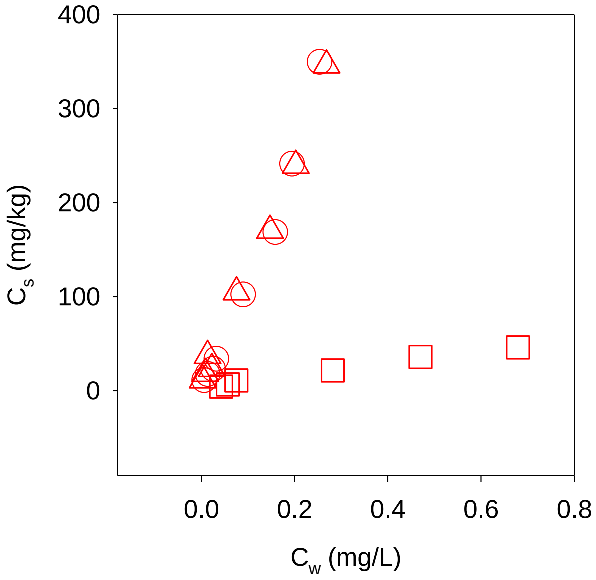


Figure B-4. Biochanin A sorption to Ca-montmorillonite (circles), Na-montmorillonite (squares), and kaolinite (triangles) at pH 8.5.

Isotherms for daidzein on clay minerals at pH 8.5 are shown in Figure S4.

Daidzein did not sorb appreciably to Na-montmorillonite so the isotherm is omitted.

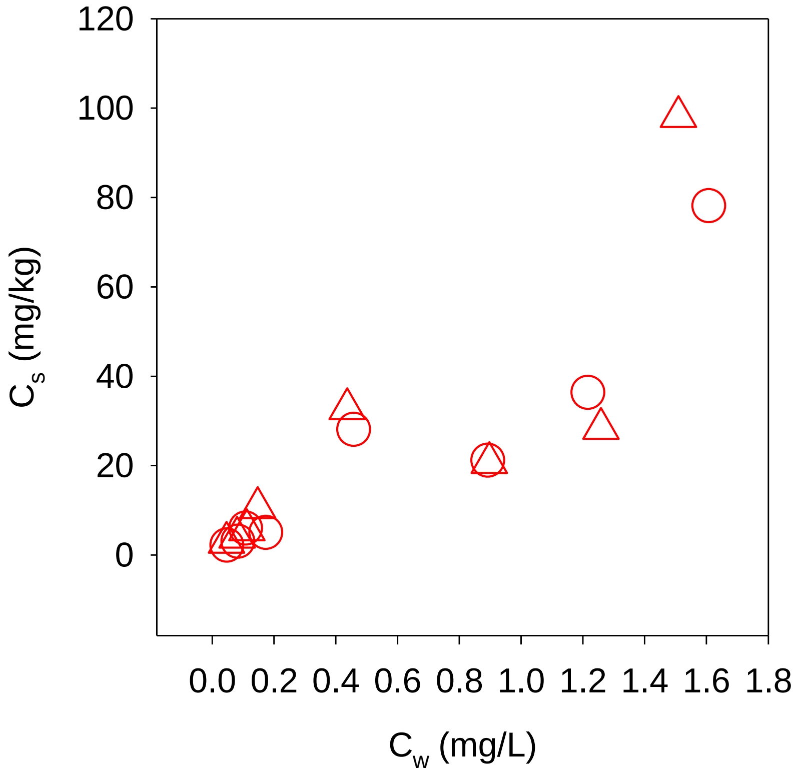


Figure B-5. Daidzein sorption to Ca-montmorillonite (circles) and kaolinite (triangles) at pH 8.5.

Isotherms for genistein, daidzein, and formononetin on Mississippi River sediment are shown in figure S5. All three compounds exhibit linear isotherms. Formononetin sorbs the most strongly, genistein the next most strongly, and daidzein the least strongly.

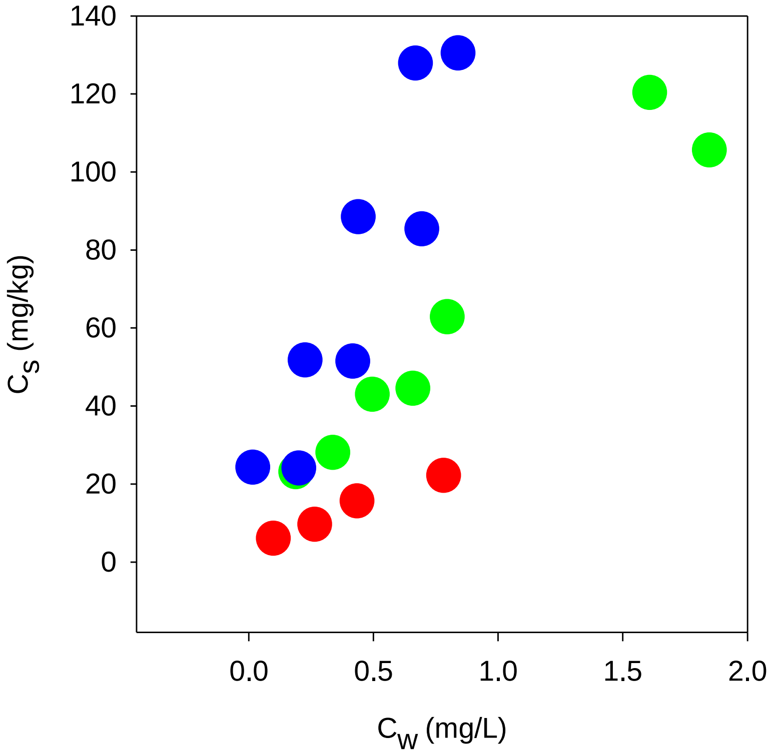


Figure B-6. Genistein (green), daidzein (red), and formononetin (blue) sorption to Mississippi River sediment, pH 8.5.

Appendix C. Supporting information for Chapter 4

Methods

Table C-1: Nitrification reactor media

Chemical	Formula	Concentration mg/L
Sodium Phosphate	Na_2HPO_4-$7H_2O$	3000
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	$(NH_4)_2SO_4$	1000

Table C-2: Synthetic Sewage media used in bioreactor

Chemical	Formula	Concentration mg/L
Bacto Peptone	-	300
Sodium Acetate	CH_3COONa	100
Ammonium Chloride	NH_4Cl	57
Ammonium Bicarbonate	NH_4HCO_3	60
Potassium Phosphate	KH_2PO_4	44
Potassium Busulphate	$KHSO_4$	34
Sodium Bicarbonate	$NaHCO_3$	394
Calcium Chloride	$CaCl_2$	220
Magnesium Sulphate	$MgSO_4$-$7H_20$	150
Ferric Chloride	$FeCl_3$-$6H_2O$	20
Alum	$Al_2(SO_4)_3$-$18H_2O$	20
Trace Element Solution	-	2 mL

Table C-3: Concentrated trace element solution used in bioreactor

Chemical	Formula	Concentration [g/L]
Citric Acid	-	2.73
Hippuric Acid	-	2
EDTA	Na_3EDTA-4H_2O	1.5
Ferric Chloride	$FeCl_3$-6H_2O	1.5
Boric Acid	H3BO3	0.25
Zinc Sulfate	$ZnSO_4$-7H_2O	0.15
Manganese Chloride	$MnCl_2$-2H_2O	0.12
Copper (II) Sulfate	$CuSO_4$	0.06
Potassium Iodide	KI	0.03
Sodium Molybdate	Na_2MoO_4-2H_2O	0.03
Cobalt Chloride	$CoCl_2$-6H_2O	0.03
Nickel (II) Chloride	NiCl2-6H20	0.03
Sodium Tungstate	$NaWO_4$-2H_2O	0.03
Water	-	1L

Table C-4. Concentrations of phytoestrogens measured (mean ± st. err.; n=5) in the ethanol blank, in the reactor prior to biodegradation, and after the biodegradation in the samples used to test the effect of fish exposure to the biodegraded phytoestrogens.

Treatment	Genistein	Daidzein	Formononetin
EtOH	18±1.1ng/L	nd	8.8±0.3ng/L
Mix	130±33ng/L	70±31ng/L	274±73ng/L
Bioreactor	34±15	nd	8.6±0.4ng/L

Results

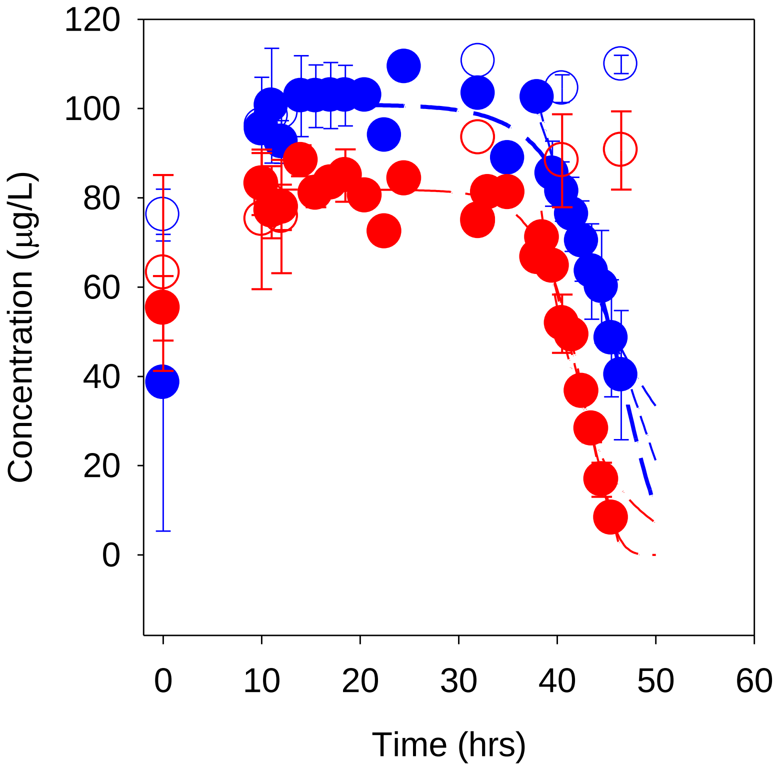


Figure C-1. Degradation of genistein (red) and daidzein (blue) in Mississippi River water collected on 6-26-13, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

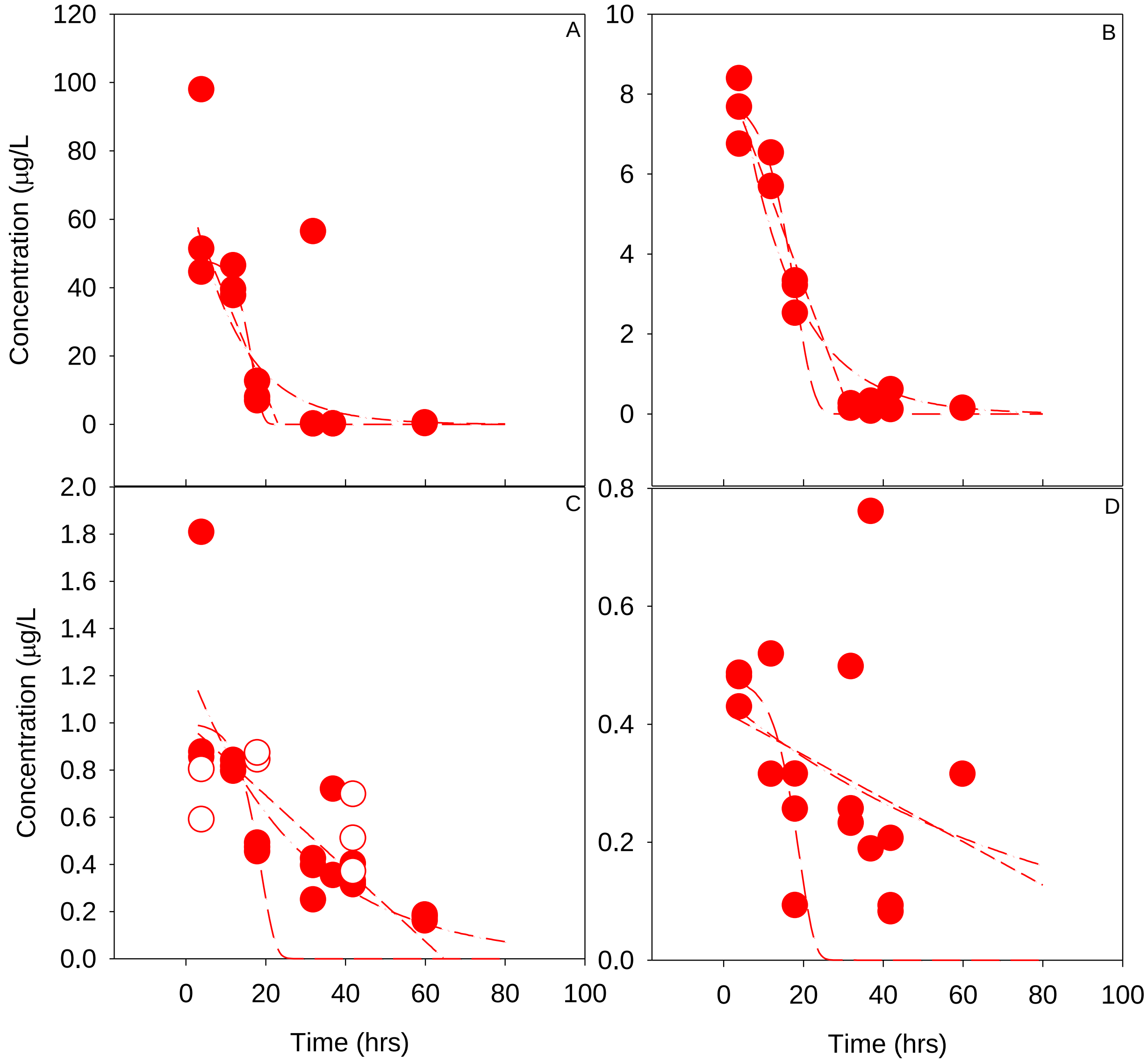


Figure C-2. Degradation of genistein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on 6-26-13, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

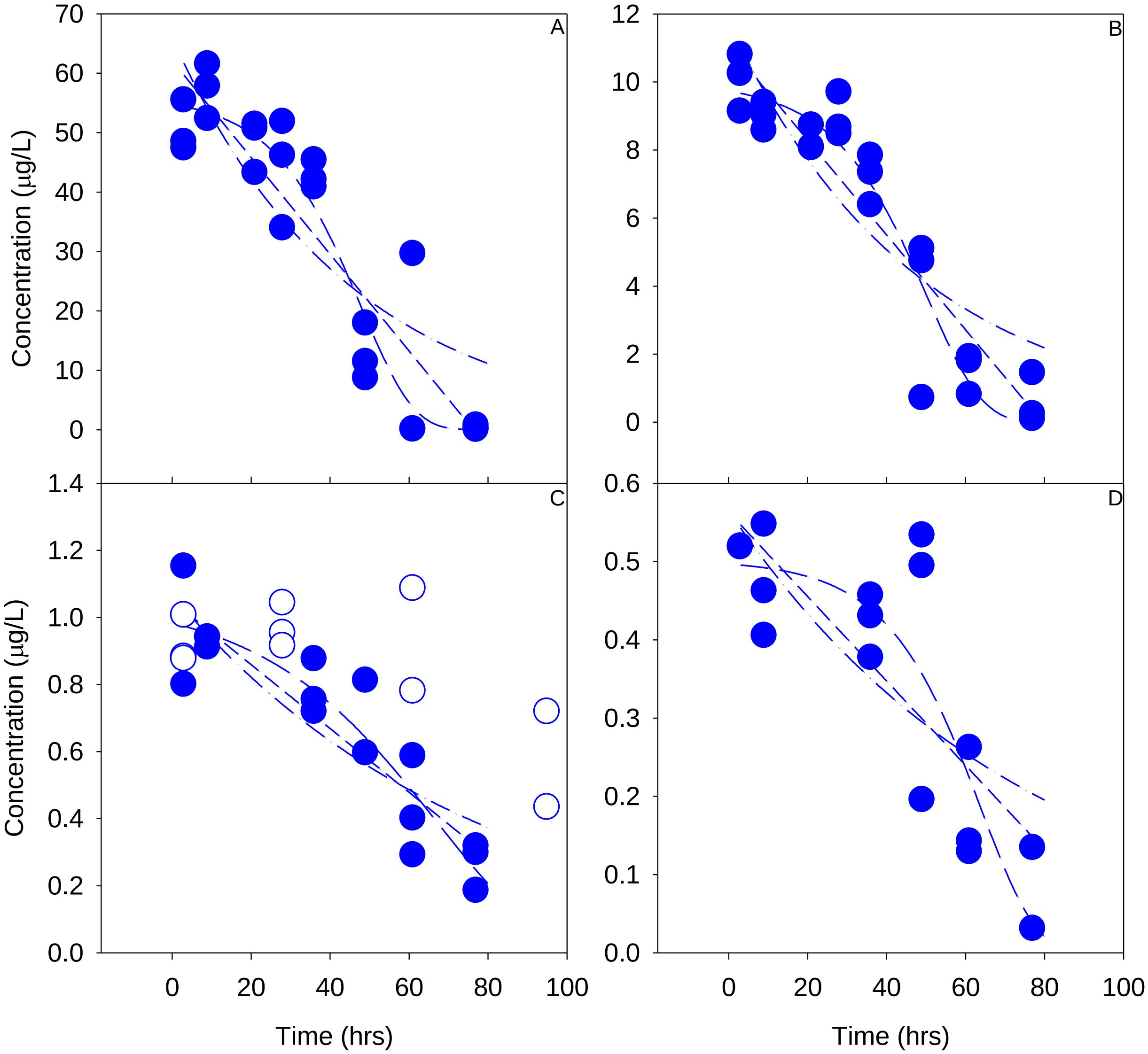


Figure C-3. Degradation of daidzein with an initial concentration of 50 µg/L (A), 10 µg/L (B), 1 µg/L (C), and 0.5 µg/L (D) in Mississippi River water collected on 6-26-13, incubated at 20° C. Long-dash lines represent fits to the Gompertz equation, short-dash lines to zero-order kinetics, and dash-dot lines to first-order kinetics. Empty circles represent controls.

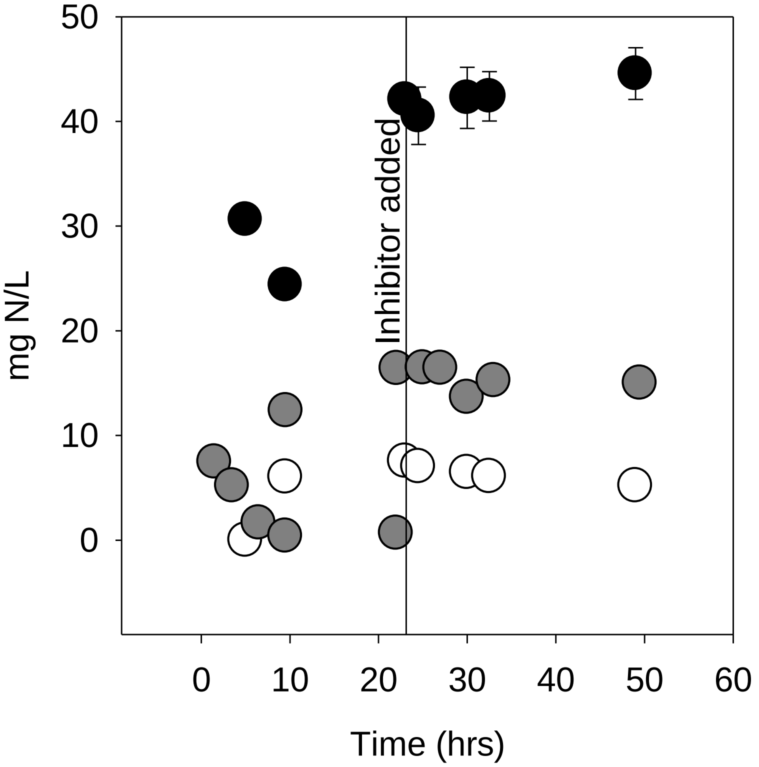


Figure C-4. Concentrations of nitrite (black), nitrate (white), and ammonia (grey) in an enriched culture of nitrifying organisms before and after the addition of allylthiourea.

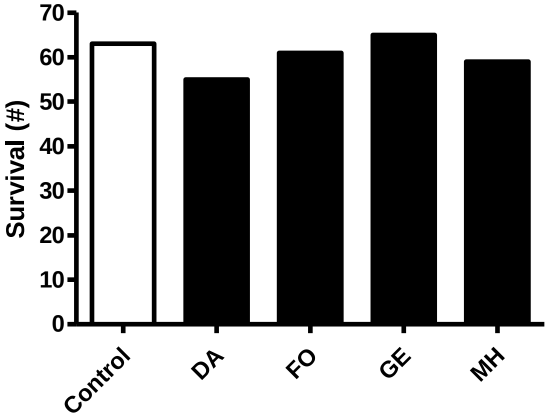


Figure C-5. Larval Fathead minnow survival following 21-day exposure to the degradation mixture. Initial cohorts were established using 75 larvae per treatment. Survival represents number of individuals remaining after behavioral testing. Significance was tested relative to control survival using a two-tailed Fisher's exact test ($p<0.05$), with significance represented by letters.

Phytoestrogen Degraded Larval Exposure

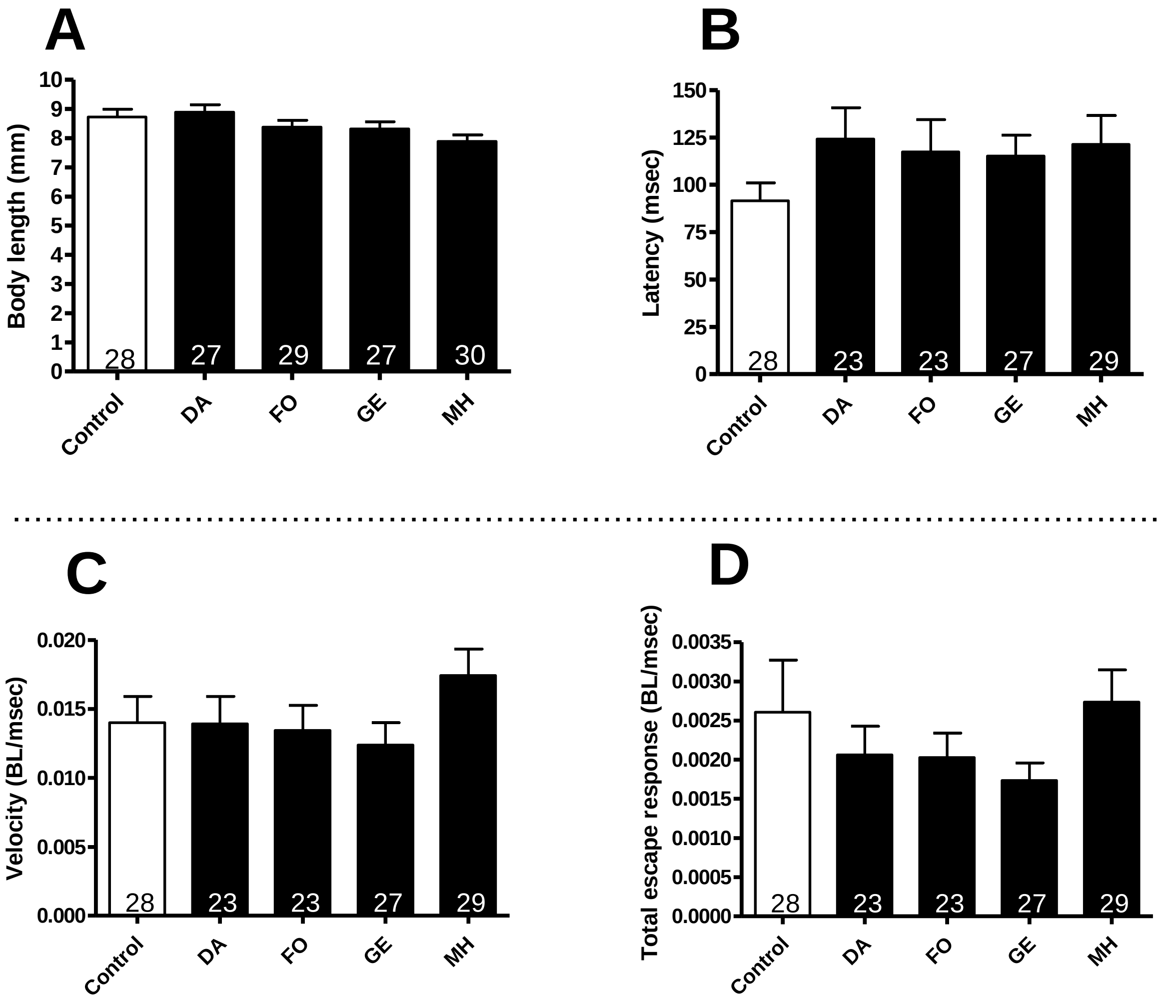


Figure C-6. Larval fathead minnow escape performance following 21-day exposure to degraded phytoestrogen compounds. C-start escape performance was quantified using. **(A)** body length (mm); **(B)** mean latency (ms) from stimulus to response; **(C)** mean escape velocity relative to body length (BL/ms); **(D)** mean total escape performance (BL/ms) defined as (distance travelled/BL)/(40+latency). Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn's post-test were used for non-normal distributions.

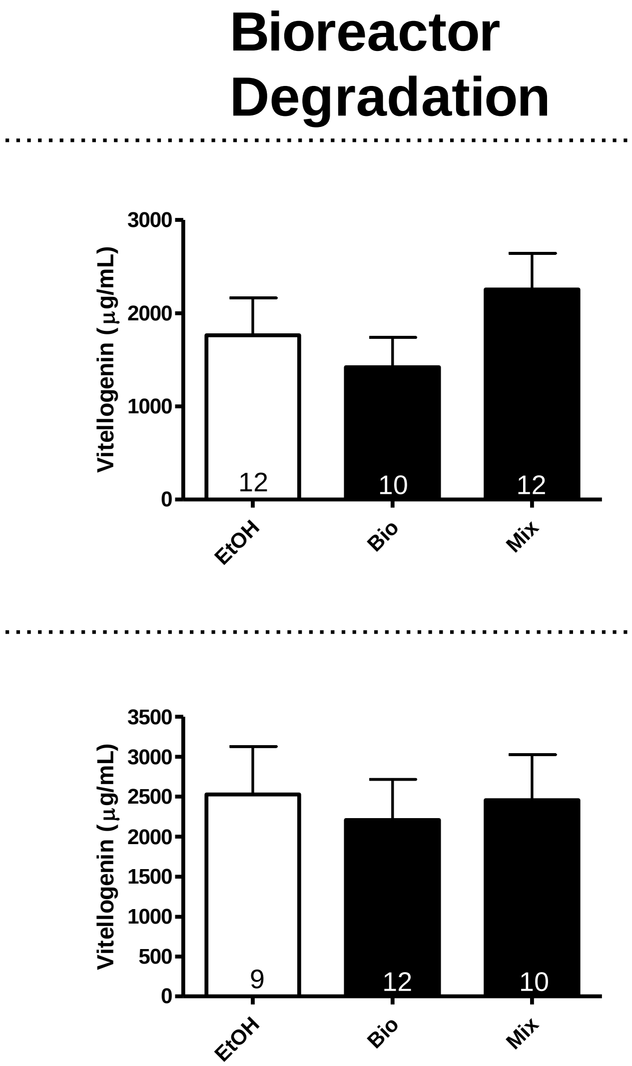


Figure C-7. Plasma vitellogenin concentrations (μg/mL) in male (top) and female (bottom) fathead minnows exposed for 21- days to the degraded phytoestrogen mixture (Daidzein, Genistein, and Formononetin 1000 ng/L per compound). Sample size for each treatment is listed in each column, with letters indicating significance (ANOVA with Tukey's post-test). Graphical representation contains quantified values (mean± standard error). Statistical analysis was performed using log transformation.

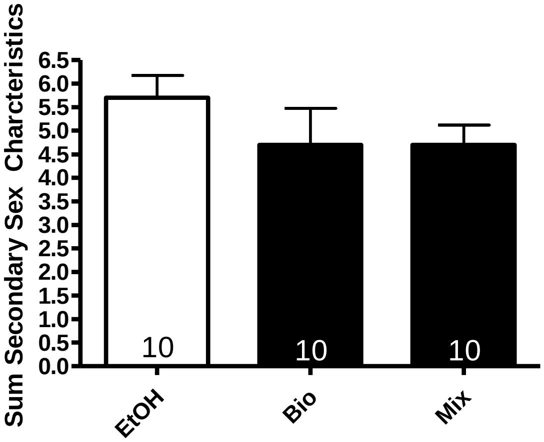


Figure C-8. Sum of secondary sex characteristics (mean ± standard error) in male fathead minnows (coloration, dorsal pad, and tubercles) exposed to the degradation mixture. Characteristics were rated individually on a 0-3 scale (absence to highly prominent) and summed to provide total expression. Sample size for each treatment is listed in each column. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn's post-test were used for non-normal distributions.

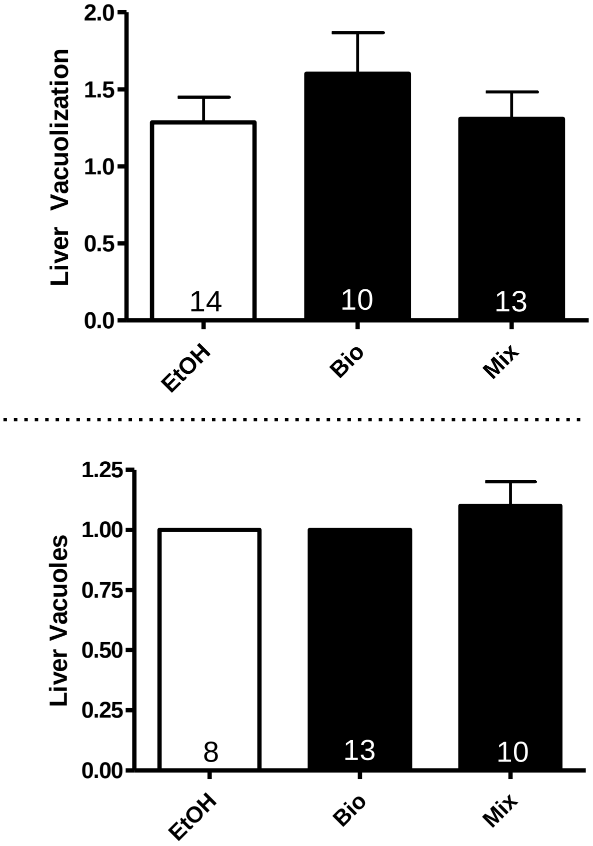


Figure C-9. Liver histological data (mean ± standard error) for male (top) and female (bottom) fathead minnows exposed 21-days to the degradation mixture. Liver sections were evaluated for vacuolization on a 0-4 scale with low values representing the absence of vacuoles. Sample size listed within mean columns. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn's post-test were used for non-normal distributions.

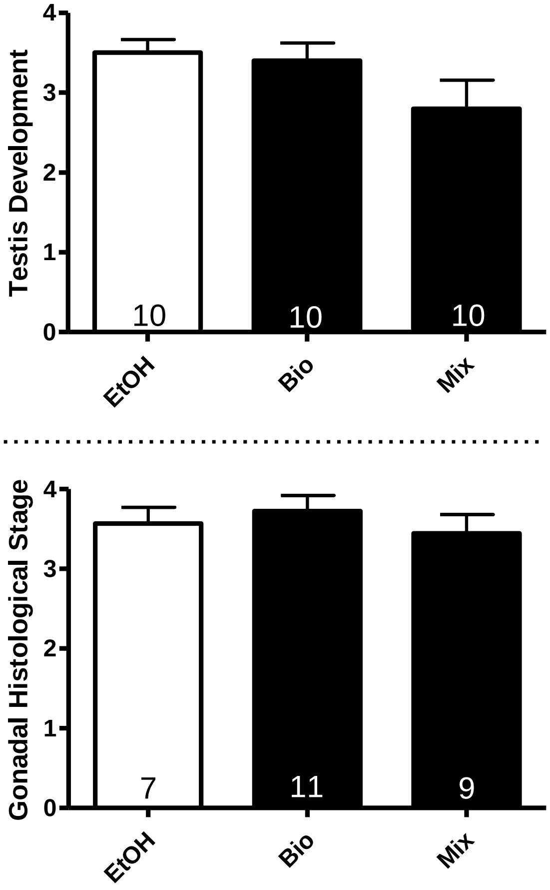


Figure C-10. Gonadal histological data (mean ± standard error) for male and female fathead minnows exposed 21-days to the degradation mixture. Gonadal sections were evaluated for developmental stage on a 0-4 scale (immature to fully mature). Sample size listed within mean columns. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn’s post-test were used for non-normal distributions.

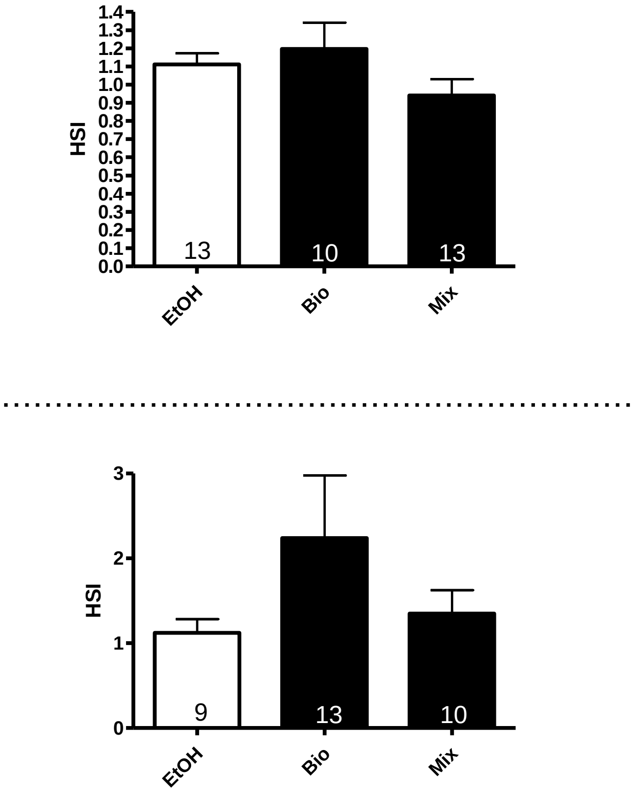


Figure C-11. Hepatosomatic Index (HSI) of male (top) and female (bottom) fathead minnows exposed 21-days to the degradation mixture. Sample size listed within mean columns. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn’s post-test were used for non-normal distributions.

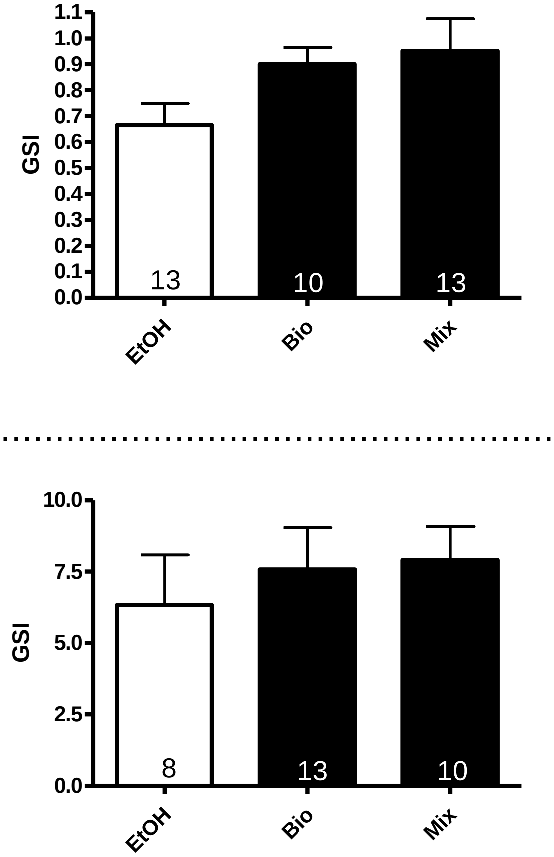


Figure C-12. Gonadosomatic Index (GSI) of male (top) and female (bottom) fathead minnows exposed 21-days to the degradation mixture. Sample size listed within mean columns. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn’s post-test were used for non-normal distributions.

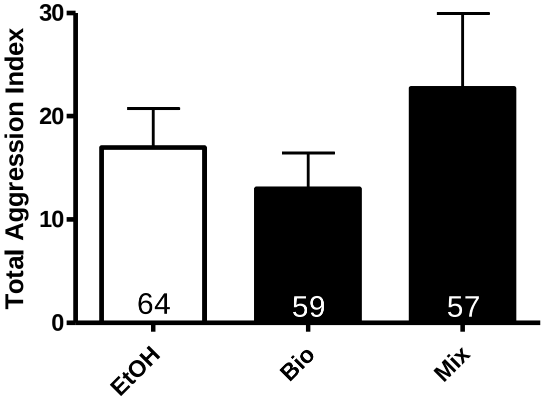


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Figure C-13. Total Aggression Index (TAI) of male fathead minnows following 21-day exposure to the degradation mixture. Males within breeding pairs were tested 3 times with 2 days between each aggression assay averaged above. TAI was calculated by dividing the number of attacks (multiplied by a factor of 10 to equally weigh parameters) by male latency to first attack. No response was defined as 300s and 1 attack to calculate TAI. Sample size for each treatment is listed in each column. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn's post-test were used for non-normal distributions.

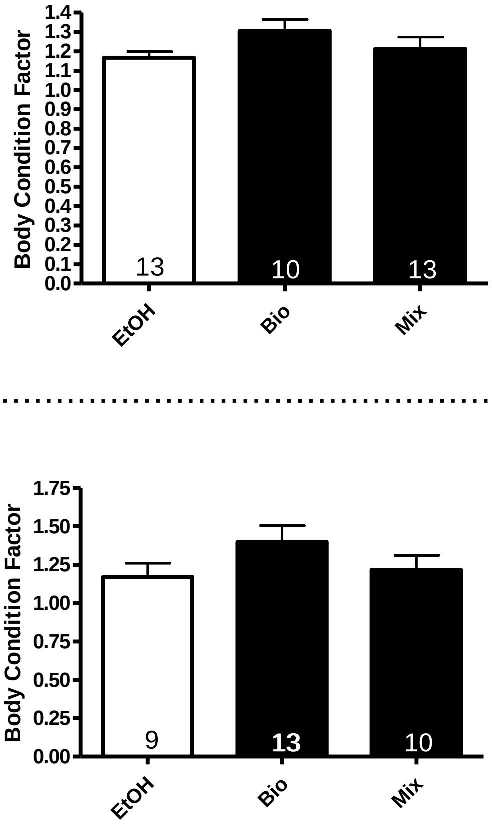

Figure C-14. Body Condition Factor (BCF) of male (top) and female (bottom) fathead minnows exposed 21-days to the degradation mixture. Sample size listed within mean columns. Data were tested for normality using a Kolmogorov-Smirnov test. One-way ANOVAs followed by Tukey Post-tests were used for normally distributed data, and Kruskal–Wallis with Dunn’s post-test were used for non-normal distributions.

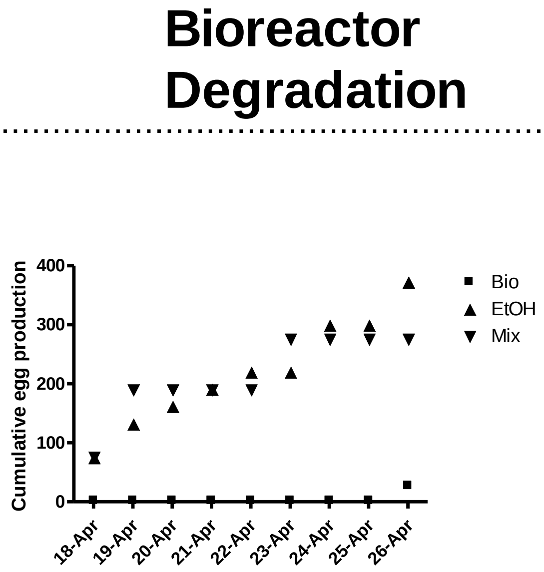

Figure C-15. Cumulative egg production of female fathead minnows exposed 21-days to the degradation mixture. Egg production was monitored over a week. Bio refers to fish exposed to the biodegradate mixture, EtOH refers to fish exposed to the ethanol control, and Mix refers to the fish exposed to the mixture of parent compounds.