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THE INFLUENCE OF SEDIMENT CHARACTERISTICS ON THE FATE OF STEROIDOGENIC COMPOUNDS IN AQUATIC SYSTEMS AND THE EFFECTS ON PROGESTERONE BIOAVAILABILITY IN A TARGET ORGANISM

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by

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Jodi L. Sangster, Ph.D.

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There is growing concern about the biologic effects stemming from steroids in impacted waterways. In aquatic systems, interaction between steroids and sediment influence both contaminant fate as well as subsequent bioavailability to aquatic organisms. The focus of this dissertation research was to gain a better understanding of steroid behavior in aquatic systems based on the physiochemical properties of sediment and to use this knowledge to better understand the biological effects stemming from sediment-associated progesterone exposure. Two natural aquatic sediments, a sand and a silty loam, were selected to represent marked differences in sediments properties. Initially, sorption of 17β-estradiol, estrone, progesterone, and testosterone was evaluated to different size fractions of each sediment to determine the steroid sorption capacity and distribution within the whole sediment. Sorption capacity was influenced more by organic carbon content than particle size; while, interactions between size fractions were found to affect the distribution of steroids within the whole sediments.

In a subsequent study, the sediments were used to evaluate the fate of progesterone and the corresponding alteration of gene expression in a target organism,

the fathead minnow (*Pimephales promelas*), using three steroid-responsive genes; vitellogenin, androgen receptor and estrogen receptor-alpha. When exposed to progesterone spiked sand, fish exhibited significant reductions in the expression of vitellogenin in the 5 and 50 ng/g treatment groups at 7 d and significant reductions in vitellogenin and androgen receptor expression after 14 d in the 50 and 500 ng/g treatment groups. In contrast, fish exposed to progesterone associated with the silty loam sediments did not show a biological response at 7 d and only realized a significant reduction in vitellogenin at 14 d in the 50 and 500 ng/g groups. In both sediments, progesterone degradation resulted in the production of androgens including androsteinedione, testosterone, and androstadienedione, as well as the anti-estrogen, testolactone. Differences in compound fate resulted in organism exposure to different suites of metabolites either in water or associated with the sediment. Results from this study suggest that environmental progestagens will lead to defeminization at environmentally-relevant concentrations, and that exposure is influenced by sediment properties.

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ACKNOWLEDGEMENTS

I consider myself to be an "accidental" graduate student in that this crazy journey was not planned. I had made the decision to go back to school to become an engineer after progressing through my first career in a biology related field. This snowballed into a master degree and now, as I prepare to enter the world as a new PhD, I am surprised by the distance I have covered. I wouldn't have dreamt of making it thus far without the help and support of numerous people along the way.

The "things" I will miss most from my time at the University of Nebraska are the people and friendships I have experienced. Some friendships have spanned multiple continents, languages, and projects. However, I am most thankful for two people in particular, the fabulous Jon Ali and Dr. Brett Sallach. Both have helped me to retain my sanity and are always quick to offer motivation and/or laughter as needed. Jon has served as a vast source of strange animal videos and interesting conversations during work in the lab and throughout numerous meals and cocktail outings. Brett has been a part of almost every academic, research, and extracurricular endeavor I have undertaken. Honestly, I don't know how I will function without his endless enthusiasm for research, life, and art nor without his vast creativity and ability to embrace distractions without (or with very little) guilt. My life is richer because of these two men and I will miss seeing them on a regular basis.

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ideas. The entire time she has been there to cheer on successes and commiserate over failures. Her dedication has served as an inspiration and allowed me many opportunities most graduate students only dream of. Additionally, I am thankful for my committee, Dr.'s Kolok, Snow, and Li, who have shared their enthusiasm for science and engineering, while challenging me with insightful questions and new ideas every step of the way. I also want to thank Arlys Blakey for always having a smile on her face, candy on her desk, and knowing how to navigate all the crazy challenges posed by the Omaha and Lincoln campuses.

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From the bottom of my heart, THANK YOU!

CHAPTER 1:CURRENT STATE OF THE SCIENCE

Sources of steroids in the environment. Estrogens and androgens have been detected in aquatic systems at concentrations that cause reproductive effects on fish and other macroorganisms. Wastewater effluent represents a significant source of natural and synthetic estrogens and androgens to the environment (Kolodziej et al., 2003; Williams et al., 2003), including the natural estrogens, estrone (E1) and 17β-estradiol (17β-E2), and the synthetic compound 17α-ethynylestradiol (EE2), used in birth control medication and hormone replacement therapy (Desbrow et al., 1998). Androgens detected in the environment include testosterone (T) and androstenedione (AD) from municipal wastewater effluents (Desbrow et al., 1998) and trenbolone acetate (TBA) metabolites in runoff from agricultural sites (Durhan et al., 2006). Progestagens have been detected frequently in wastewater influent and effluents averaging between 39.6-57 and 6.9-12.1 ng/L respectively (Fan et al., 2011; Liu et al., 2011). AD and Pr have been found concurrently in sediments in waterways receiving paper mill effluents at concentrations greater than those found in the water column. Although environmental concentrations of these compounds are typically in the low ng/L range, reproductive effects have been observed at steroid concentrations less than 1 ng/L (Jobling et al., 1996).

Animal agriculture may be a significant source of steroids in aquatic systems. Supplements and growth promotants containing steroids are routinely administered to beef cattle to increase size and rate of growth (Kolok and Sellin, 2008). Wastes excreted by cattle contain several endogenous and exogenous androgens, estrogens, and progestegens, as well as, synthetic androgens such as trenbolone (Tb). The estrogenic compound, 17β-E2, and its metabolites, E1, estriol, and 17α-E2, have been found in

runoff from cattle feedlots in Nebraska at concentrations at or below 2600 ng/L and in fresh manure at average concentrations as high as 25.6 ng/g (Bartelt-Hunt et al., 2012). The same study reported frequent detection of the androgens, T, AD, and androsterone in runoff from cattle feedlots at maximum concentrations for each as 475, 1050, and 24,300 ng/L respectively. However, T was only detected in fresh manure at one sampling time at a concentration of 1.5 $\pm$ 0.05 ng/g while AD was frequently detected in fresh manure at average concentrations between 0.5 – 5.3 ng/g (Bartelt-Hunt et al., 2012). Another study reports between 0.5 and 150 ng/g T and 2.6 – 200 ng/g AD in manure leachate in rangeland cattle (Kolodziej and Sedlak, 2007). Furthermore, the synthetic androgen Tb has been documented in manure with the 17α-Tb isomer found at concentrations up to 75 ng/g in fresh manure, while the 17β-Tb isomer is typically below 5 ng/g (Schiffer et al., 2001; Schiffer et al., 2004). It has been shown Tb and trendione (TND) are persistent in liquid manure with half-lives of both isomers greater than 250 days (Schiffer et al., 2001). It has been estimated cattle excrete 5-30 times more progestagens than either estrogens or androgens with up to 390 ng/g in manure depending on sex and reproductive phase of the animal (Lange et al., 2002). Average Pr concentrations in fresh manure of 0.6 - 56 are more commonly reported from feedlot and rangeland cattle (Bartelt-Hunt et al., 2011; Bartelt-Hunt et al., 2012; Kolodziej and Sedlak, 2007). However, some studies suggest that these values may be low due to excretion of conjugated forms which are not readily detectable (Mansell et al., 2011). This may be of concern as manure is commonly land applied to agricultural fields as a source of fertilizer or may runoff from feedlots during storm events.

Fate of steroidogenic compounds in agricultural soils. Several studies have attempted to gain insight into the behavior of steroidogenic compounds in agricultural systems. Steroids are moderately to highly lipophilic with K_d values for estrogens ranging between 14 – 170 mL/g and is strongly correlated to the organic content of the soils tested (Sarmah et al., 2008). Organic carbon normalized values, log K_{oc}, have been found for estrogens ranging between 2.36-4.13 (Casey et al., 2005; Sarmah et al., 2008). Values of log Koc for AD and T have been found to be as high as 7.79 when samples had longer equilibration times and soils were sterilized (Kim et al., 2007). While Pr has not been widely evaluated, one study found log Koc to range between 3.57 – 5.53 at neutral pH for Pr associated with 3 types of bulk organic matter (Neale et al., 2009) and between 2.97 and 3.4 for levonorgestrel, a synthetic progestin, in five agricultural topsoils (Tang et al., 2012). Additionally, the presence of dissolved organic carbon (DOC) reduces estrogen sorption to soil particles and suggests higher mobility of the compound in the environment (Stumpe and Marschner, 2010).

Several studies have suggested that steroid transport in agricultural systems may be due to a strong association with colloidal material within natural soils (Casey et al., 2003; Casey et al., 2008; Schiffer et al., 2004; Mansell et al., 2011). Field studies suggest that steroid transport may be enhanced by organic matter content of the soils, as well as increased persistence due to saturated conditions (Casey et al., 2008). Additionally, transport may be enhanced by disturbances and naturally occurring macropores within the soil column (Casey et al., 2005; Gall et al., 2011) as evidenced by increased steroid concentrations in subsurface tile drainage systems located below manure treated fields after irrigation, precipitation, and during periods of snowmelt (Arnon et al., 2008; Gall et

al., 2011). Additional research suggests that both steroid degradation and sorption processes should be considered when determining risk associated with steroid transport through the soil column (Casey et al., 2004).

Effects of particle size on steroid sorption. While several studies have explored the fate and transport of steroidogenic compounds in agricultural systems, relatively little is known about the effects of soil or sediment particle sizes on steroid fate in the environment. Existing studies focus on interactions between estrogens and colloids (Yamamoto and Liljestrand, 2003; Zhou et al., 2007) or dissolved organic matter (Yamamoto et al., 2003) or sorption of 17β-E2, E1, and T to select size fractions within a whole soil/sediment (Duong et al., 2010; Gineys et al., 2012) or specific mineral constituents (Bonin and Simpson, 2007) and are unable to provide clear insight into how physical characteristics of various particle size fractions may influence sorptive behavior. Sun et al. (Sun et al., 2012) systematically evaluated the sorptive behavior of a synthetic estrogen, 17α-ethinyl estradiol (EE2), and two other endocrine active compounds, BPA and phenanthrene, in soil and sediment particle size fractions and determined sorption of all three compounds was strongly correlated with the organic carbon (OC) content of the soil. Recently, Qi et al. (2014) evaluated sorption behavior of T in five fractions from an agricultural soil. Results show T has a higher affinity for smaller particle size fractions which is likely due to both increased surface area and OC content in the smaller particle size fractions. While studies like these provide valuable insight into the sorptive behavior of soils, each of these studies used soils or sediment fractionated prior to sorption testing, which may not represent how steroids would naturally distribute between particle sizes within whole sediments. This may be of greater concern in aquatic systems where

sediments are susceptible to near constant mixing due to stream flow and bioturbation from aquatic organisms.

Microbial degradation of steroids. The degradation of steroids in agricultural systems appears to be primarily microbially mediated under aerobic conditions (Das et al., 2004; Fan et al., 2011; Khan et al., 2008; Ying and Kookana, 2005) with reported half-lives of E2 ranging between 0.17 days to 3 days in soils (Xuan et al., 2008; Ying and Kookana, 2005) and between 0.3 to 7.3 days for T (Lee et al., 2003). Degradation rates ranging from 0.003 to 0.036 h^{-1} for E2 and between 0.004 to 0.072 h^{-1} for T (Das et al., 2004) have been documented. Experiments evaluating levonorgestrel, a synthetic progesterone, have documented half-lives between 4.32 and 11.55 days (Tang et al., 2012). Additional research has shown steroids to degrade more rapidly in soils with increasing moisture content and with increasing temperatures between 15-25^{o}C (Xuan et al., 2008). However, the presence of some veterinary antibiotics in manure amended soils may significantly slow the degradation of steroids (Chun et al., 2006; Xuan et al., 2008). Increases in organic carbon content of soils has been found to decrease E2 mineralization in soils and may affect sorption of estrogens to soil particles (Stumpe and Marschner, 2010; Stumpe and Marschner, 2009). It has been suggested the bonding of estrogens to organic carbon either masks the molecular structure from or becomes too large for uptake by enzymes present in the soil.

Studies detailing the degradation of steroids in natural systems do not typically evaluate the type of microbes present or changes in microbial populations over time. However, it is possible to consider major microbial processes that may affect steroid fate in soil including hydrolysis, hydroxylation, oxidation, ring cleavage, sulfioxidation,

among other reactions (Loffredo and Senesi, 2006). Several studies have evaluated progesterone degradation by species of bacteria including *Aspergillus, Anthrobacter, Chlorella, Penicillium, Bacillus,* and *Streptomyces* (Bartmanska et al., 2005; Hunter and Carragher, 2003; Hunter et al., 2009; Mostafa and Zohri, 2000; Pollio et al., 1996; Yildirim et al., 2010). While studies differ on degradation rates and the number of intermediate products found, most studies agree that Pr may exhibit interconversion with hydroxyprogesterone but actual pathways for degradation show progesterone will eventually biotransform to testololactone by a combination of hydrolyzation and oxidation processes (Hunter and Carragher, 2003; Hunter et al., 2009). Testololactone is thought to exhibit antiestrogenic properties through aromatase inhibition (Seralini and Moslemi, 2001). Intermediate products reported during degradation may include testosterone acetate, T, hydroxytestosterone, and/or AD (Figure 1.1). However, it is important to note that Pr degradation has not been evaluated in more complex environmentally relevant systems.

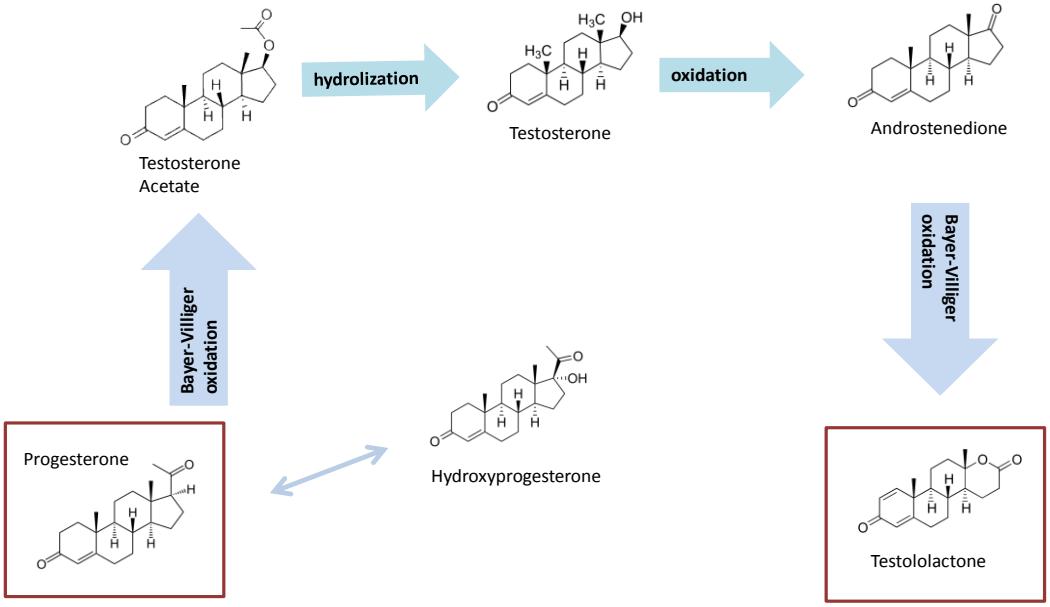


Figure 1.1. Suggested biotransformation products and mechanisms for progesterone degradation (Hunter and Carragher, 2003; Hunter et al., 2009)**.**

Fate of steroidogenic compounds in aquatic systems. The role of sediment in the fate of steroidogenic compounds in aquatic systems is not clearly understood, and estrogens and androgens may exhibit different behavior in the environment. Field studies have indicated that sediments are an environmental sink for estrogens in aquatic systems (Braga et al., 2005; Peck et al., 2004; Ying et al., 2002; Ying and Kookana, 2005), although there is evidence that steroids may desorb from the solid phase to water with migration of steroids dependent on physical properties of a particular sediment (Labadie et al., 2007). To date, there have been a very limited number of studies evaluating steroid transformation in sediment, and results from these studies indicate steroids bound to aerobic and anaerobic sediments have significantly different half-lives. For example, half-lives for natural estrogens in aerobic sediment have been reported to range from as

little as 1 day to > 4 days (Jurgens et al., 2002; Labadie et al., 2007; Robinson and Hellou, 2009). In contrast, half-lives ranging from 14 to 70 days have been reported for natural estrogens in anaerobic sediment (Robinson and Hellou, 2009; Ying et al., 2002). Additional studies have shown increased sorption and mineralization of some endocrine active compounds in stream sediments (<7d) when compared to natural biofilms (<180d) present in the system(Writer et al., 2011). Although reported half-lives for some steroids, such as 17β-E2 and E1, in sediment are relatively short, the degradation pathways for these compounds are not well-described and the formation of biologically-active metabolites does occur. E1 is formed from biodegradation of 17β-E2 (Johnson et al., 1998), and recent studies of 17β-E2 degradation in sediment have determined that additional intermediate products may be formed with chemical structure similar to that of E1 (Robinson and Hellou, 2009). Therefore, while parent steroids may be degraded in natural systems, endocrine-active metabolites may be present for significantly longer time periods.

Biological effects of aqueous steroids. Conventionally, observed biological effects in aquatic organisms are linked to concentrations of steroids in the aqueous phase. Since steroid hormones do not bioaccumulate, but rather are metabolized within the organism and excreted in water soluble form (Kolok and Sellin, 2008), it is common to evaluate bioindicators of steroid exposure rather than the concentration of the steroid or its metabolites in the blood or tissue of the aquatic organism. One of the most effective and widely used biomarkers of exposure of fish to estrogenic compounds is the inappropriate expression of vitellogenin (Vtg), an egg yolk precursor protein. Vtg is produced in female fishes in response to endogenous estrogen production. Fish exposed to steroidogenic

compounds are susceptible to measurable alterations in the expression of Vtg. Several studies have shown steroids to cause a non-monotonic dose response in targeted organisms. Male fathead minnows exposed to 17β-E2 have been shown to significantly exhibit inappropriate levels of Vtg at aqueous concentrations as low as 28 ng/L (Seki et al., 2006). Alternately, female fathead minnows exposed to 17β-Tb have exhibited significant reductions in the expression of hepatic vtg at concentrations as low as 27 ng/L (Ankley et al., 2003). While some metabolites of the parent compound have been shown to have less biological strength in the fathead minnow (Shappell et al., 2010), they are still biologically active in the test organism at environmentally relevant concentrations.

Biological effects of aqueous progesterone. Atteke et al. (2003) evaluated the effects of progesterone alone and in combination with 17β-E2 on the reproductive axis of immature rainbow trout (*Oncorhynchus mykiss*). Fish were injected with a single dose of 17β-E2, Pr, or Pr and 17β-E2 at a rate of 1.5mg/kg and sacrificed from 48 to 72 hours after injection depending on the experimental group. Data indicate metabolic conversion of 17β-E2 to estrone and that of progesterone to 17-hydroxyprogesterone (17 OH-P) within the blood plasma of the study animals within 72 hours. Exposure to Pr alone was not found to affect hepatic Vtg or rtER nor did it enhance 17β-E2 effects on either gene. Alternatively, at the pituitary level, Pr does exhibit a strong stimulating effect. Overall, results suggest that Pr may be cleared at a much higher rate than 17β-E2 in the study animals. Additionally, 17β-E2 seems to be poorly metabolized while Pr appears to be actively transformed to 17 OH-P. This study also suggests Pr may be metabolized into 17β-E2 in the brain at levels that will produce estrogenic effects but low enough to not induce changes in peripheral blood concentrations. The lack of synergistic or cumulative

effects in the target organism suggests that 17β-E2 and P may act through similar pathways.

DeQuattro et al. (2012) evaluated the effects of aqueous concentrations of Pr (0-1000 ng/L) on both male and female fathead minnows. Fish were exposed for 21 days with 2 females and 1 male in each group. Study animals were evaluated for gonadal somatic index (GSI), ovarian cortisol level, hepatic Vtg, and embryonic development. While no changes in these metrics were seen in male fish exposed to the steroid, female fish did see a significant reduction in fecundity with aqueous concentrations of 100 to 1000 ng/L and increased GSI at 1000ng/L Pr. However, this did not affect the rate of fertilization only the success. During the exposure, male fish did not express inappropriate levels of Vtg. However, female fish exposed to 10 to100 ng/L had significant reductions in the expression of Vtg.

More recently, studies have attempted to gain an understanding of the transcriptional response to Pr exposure seen in fathead minnows using *in vitro* and *ex vivo* studies focused on the ovary (Chishti et al., 2014), testis (Chishti et al., 2013), and specific signaling pathways involving nuclear Pr and androgen receptors (Ellestad et al., 2014). In an ex vivo study using fathead minnow ovaries incubated for 6 to 12 hours, Pr exposure increased the production of T but not 17β-E2 at both 6 and 12 hours resulting in significant reductions in the expression of androgen receptor (AR) and estrogen receptor –α (er-α) at 6 hours with no significant differences detected between the negative control and either receptor after 12 hours (Chishti et al., 2014). A previous study using fathead minnow testis also documented and increase in T production at 6 hours but not after 12 hours *in vitro* with no significant difference found in the expression of estrogen or

androgen receptors evaluated (Chishti et al., 2013). While transcriptional activation assays evaluating nuclear Pr and androgen receptors originating from female fathead minnows show that Pr increased activation in the nuclear Pr receptor while acting as a weak AR agonist (Ellestad et al., 2014).

Biological effects of sediment-associated steroids. There is a growing body of work indicating sediments may act as a source of biologically active compounds in aquatic systems. Caged fish deployed downstream of waste water treatment plants have been shown to exhibit reduced expression of Vtg suggesting estrogenicity in the aquatic system (Jobling et al., 1996). However, these studies do not differentiate between aqueous and sediment-associated steroids within the aquatic system. Few studies have evaluated the importance of sediment-associated compounds. Natural sediments from agriculturally impacted areas have been shown to act as a source of endocrine active compounds in fathead minnows (Jeffries et al., 2011; Sellin et al., 2010). However, it was not possible to determine what compound or suite of compounds caused the effects seen in the fish. Further testing suggested an anti-estrogen was present in the sediments (Jeffries et al., 2011). A different study suggests estrogens show preferential sorption to smaller particle size fractions of a natural sediment (Duong et al., 2010) and that estrogens associated with smaller particles (<1 μm) exhibit a greater degree of bioavailability in Japanese medaka (Duong et al., 2009). However, these studies did not evaluate whole sediments and was spiked such that the steroid may or may not have been truly sediment-associated in the system. Additionally, steroid fate was not evaluated as it relates to bioavailability.

Recently, attention has been given to elucidating the role of whole sediment composition on both bioavailability and compound fate in aquatic systems. As detailed in previous sections, sediment composition will affect the degree of desorption of steroids (Qi et al., 2014; Sun et al., 2012), as well as, the degradation rates and metabolites formed during microbial degradation of the parent compound (Khan et al., 2008; Khan and Lee, 2010; Mashtare et al., 2013). Therefore, it is possible sediment composition may affect not only the concentration, location in the sediment-water system, and specific contaminants present, but also affect the mechanism of exposure in fish as it is not clear if biological effects stem from desorption of steroids from the sediment and/or direct contact between the fish and sediment-associated steroid. Sangster et al. (2014) found both the biological strength of the parent compound and metabolites formed, as well as, the sediment type as it affects the persistence of compounds will induce different responses in fish exposed to either 17β-E2 or 17β-Tb associated with sand or silty loam sediment. Additionally, differential bioavailability was observed between fish exposed to 17β-Tb as a function of sediment type. This, as well as, other studies have shown limited detection of aqueous phase steroid (Jeffries et al., 2011; Sellin et al., 2010) suggesting direct contact may at least be partially responsible for the observed biologic effects. Additionally, Zhang et al. (2015) evaluated agrichemical fate and bioavailability in field-based mesocosms within an agriculturally-influenced watershed and determined that exposure to river sediment was necessary for the observed biologic response in fathead minnows. Recently, Jessick et al. (2014) attempted to evaluate the mechanism by which sediment-associated 17β-Tb is bioavailable to female fathead minnows. It was determined that direct contact with whole sediments was not necessary for

defeminization of fish to occur. It was not possible to determine conclusively if effects seen in the fish were caused by 17β-Tb associated with a small quantity of fine particles in the water column or by free steroid that had desorbed from sediments.

The following dissertation will use two natural sediments to gain a better understanding of steroid behavior in aquatic systems and the subsequent bioavailability in a target organism. The sediments were selected to represent marked differences in physiochemical characteristics. Sorption experiments were performed using steroids commonly found in agricultural areas; 17β-E2, E1, T, and Pr. These experiments will look at steroid sorption to whole sediments, in addition to, sorption capacity and preferential sorption to particle size fractions within each sediment. This information will be applied to a more complex system to evaluate the fate of Pr and bioavailability to female fathead minnows.

CHAPTER 2: PHYSIOCHEMICAL CHARACTERISTICS OF SEDIMENTS

Natural aquatic sediments were collected from two sites representing a wide range in physical properties and include: 1) a silty loam collected from Plum Creek in Seward, Nebraska; and 2) a sandy sediment from the Elkhorn River near Winslow, Nebraska. These locations were selected as steroid hormone contamination had not previously been detected at these sites (Sellin et al., 2009), nor were the untreated sediments found to induce endocrine disrupting effects in previous laboratory studies (Jessick et al., 2014; Sangster et al., 2014). Sediments from these locations have been used frequently in laboratory and field studies either directly (Jessick et al., 2014; Sangster et al., 2015; Sangster et al., 2014) or in close proximity to the collection sites used in this study (Jeffries et al., 2011; Sellin et al., 2009; Sellin et al., 2010). Sediment was collected from the top 10 cm of the streambed using shovels and was stored in 5 gal buckets until use. While the same two sediments were used throughout the projects detailed in this dissertation, specific preparations prior to use in each experiment differed and are explained in the following chapters.

Table 2.1. Selected soil properties of whole sediments and detailed particle size fractions used in sorption capacity experiments.

Sediment Classification[a]	Fraction	Particle Size[b]	Surface Area[c]	Organic Carbon[d]	Cation Exchange Capacity[e]	Dominant Clay Type[f]
		µm	m^2/g	%	m-eq/100g	
Silty Loam	Whole		19.9	2.55	16.6	smectite
	Sand	> 53	14.2	2.1	4.0	
	Silt, Large	17.7 ± 0.097	4.55	0.87	3.3	
	Silt, Small	8.1 ± 0.06	6	1.45	4.6	
	Clay	1.43 ± 0.22	41.7	6.39	29.3	
	Colloid	0.87 ± 0.14	47.9	4.17	42.3	
Sand	Whole		1.36	0.23	4.8	illite
	Sand, Large	> 106	0.98	0.17	0.1	
	Sand, Small	106-53	3.9	0.44	3.1	
	Fines	<53	7.9	0.9	4.4	

[a]Determined by Midwest Laboratory Inc., Omaha, NE, using hydrometer analysis. [b]Measured using sieve or zetasizer. [c]BET analysis performed by Particle Technology Labs, Downers Grove, IL. [d] Microwave digestion (Islam and Weil, 1998). [e] Compulsive exchange method (Dane and Topp, 2002). [f] XRD analysis performed by KT-Geo, Gunnison, CO.

Select properties of each whole sediment and particle size fractions thereof are presented (Table 2.1). Sediments were classified as sand (92% sand, 6% silt, 2% clay) and silty loam (16% sand, 60% silt, 24% clay) by Midwest Laboratory Inc. (Omaha, NE) using sieve and hydrometer analysis and show marked differences in surface area, organic carbon content, and cation exchange capacity. Additionally, mineralogy varies between the two sediments (Table 2.2) with marked differences in total phyllosilicate (clay) content of the whole sediment and in the composition of phyllosilicates where illite as the dominant fraction in the sand (65.1% of phyllosilicates, 2.8% of the total

sediment) and the silty loam is predominantly interlayer smectite (54.7% of phyllosilicates, 7.2% of the total sediment).

Table 2.2. Results of XRD analysis of whole sediments.

	Sand	Silty Loam
Whole Rock Mineralogy (Weight Percent)		
Quartz	70.0	67.1
K-Feldspar	6.0	2.6
Plagioclase	18.4	12.8
Calcite	1.3	0.2
Pyrite	0.0	0.3
Total Phyllosilicates	4.3	17.0
TOTAL	100.0	100.0
Phyllosilicate Mineralogy (Relative Abundance)		
R0 M-L I/S 90S*	27.9	54.7
Illite & Mica	65.1	42.4
Kaolinite	7.0	2.9
TOTAL	100.0	100.0
Summary Mineralogy (Weight Percent)		
Quartz	70.0	67.1
K-Feldspar	6.0	2.6
Plagioclase	18.4	12.8
Calcite	1.3	0.2
Pyrite	0.0	0.3
R0 M-L I/S 90S*	1.2	9.3
Illite & Mica	2.8	7.2
Kaolinite	0.3	0.5
TOTAL	100.0	100.0

*R0 M-L I/S 90S - Randomly Ordered Mixed-Layer Illite/Smectite with 90% Smectite Layers

CHAPTER 3: THE EFFECT OF PARTICLE SIZE ON SORPTION OF ESTROGENS, ANDROGENS AND PROGESTAGENS IN AQUATIC SEDIMENT

This chapter presents data published in the following peer-reviewed publication:

Sangster JL, Oke H, Zhang Y, Bartelt-Hunt SL. The effect of particle size on sorption of estrogens, androgens and progestagens in aquatic sediment. *J. Hazard. Mater*. **2015**;299:112-121.

3.1 INTRODUCTION

In addition to being naturally occurring in the environment, large quantities of steroid hormones including 17β-estradiol (17β-E2), estrone (E1), testosterone (T), and progesterone (Pr) are used annually for both human health and animal production. Studies have shown that these compounds pose risks to fish and other aquatic organisms at extremely low concentrations (~1ng/l) such as decreased fertility, feminization, and hermaphroditism (Tetreault et al., 2011). In particular, attention has focused on the feminization of fish in surface waters receiving discharges of steroid-containing municipal wastewater effluent (Duong et al., 2009; Tetreault et al., 2011)(Kolodziej et al., 2003; Tetreault et al., 2011; Williams et al., 2003). 17β-E2, E1, T, and Pr are commonly found in wastewater effluents and in sediments and receiving water downstream of municipal wastewater discharges (Fan et al., 2011; Liu et al., 2011). More recently, attention has focused on animal production as a source of steroid hormones to aquatic environments as animals may be given growth promotants in the form of hormones. Wastes contain androgens, estrogens, and progestegens, as well as synthetic hormones such as trenbolone (17β-Tb) derived from endogenous and exogenous sources and are

commonly land applied to agricultural fields as a source of fertilizer. Hormone transport from agricultural fields and feedlot surfaces has been documented during irrigation and precipitation events (Bartelt-Hunt et al., 2012; Gall et al., 2011; Scott Mansell et al., 2011). The presence of steroidogenic compounds in surface water and the corresponding endocrine-disrupting effects on aquatic species has been documented adjacent to animal production facilities (Jeffries et al., 2011; Sellin et al., 2009; Sellin et al., 2010). Due to their hydrophobicity, hormones will predominantly sorb to sediment which act as potential sinks and sources of hormones and exposure to hormones bound to sediment can elicit biologic effects in aquatic organisms, either by acting as a source of free hormone through desorption from the sediments and/or by direct contact between fish and sediment-associated hormone (Sangster et al., 2014). Zhou et al. (2007) found 4-26% of E1 and 15-30% of 17β-E2 downstream of a wastewater treatment plant was associated with suspended aquatic colloids. Increasing evidence suggests sediment texture and particle size may play an important role in the persistence (Stumpe and Marschner, 2010) and biologic effects (Duong et al., 2009; Jessick et al., 2014)of hormones in aquatic systems.

The effects of soil particle size on contaminant distribution has been evaluated experimentally and in the field for a variety of contaminants including trace metals (He et al., 2012) and other hydrophobic organic compounds including octylphenol, nonylphenols, bisphenol a (BPA), polychlorinated biphenyls, dibutylphthalate, polycyclic aromatic hydrocarbons, and various perfluorinated compounds (Lu et al., 2012; Pierard et al., 1996; Zhao et al., 2012). However, there have been a limited number of studies evaluating how steroid hormones distribute among various particle size fractions in

aquatic sediment. Existing studies focus on interactions between estrogens and colloids (Yamamoto and Liljestrand, 2003; Zhou et al., 2007) or dissolved organic matter (Yamamoto et al., 2003) or selected soil / sediment particle size fractions (Duong et al., 2009; Gineys et al., 2012; Duong et al., 2010a) or mineral constituents (Bonin and Simpson, 2007) and do not look at distribution among fractions within a whole soil or sediment. Sun et al. (2012) systematically evaluated the sorptive behavior of a synthetic estrogen, 17α-ethinyl estradiol (EE2), and two other endocrine active compounds, BPA and phenanthrene, in soil and sediment particle size fractions and determined sorption of all three compounds was strongly correlated with the organic carbon (OC) content of the soil. Recently, Qi et al. (2014) evaluated sorption behavior of T in five fractions from an agricultural soil. Results show T has a higher affinity for smaller particle size fractions which is likely due to both increased surface area and OC content in the smaller particle size fractions. While studies like these provide valuable insight into the sorptive behavior of soils, each of these studies used soils or sediment fractionated prior sorption testing, which may not represent how steroids would naturally distribute between particle sizes within whole sediments.

This study focuses on evaluating the sorptive behavior of four steroid hormones to different size fractions from two natural sediments. The objective was to evaluate not only sorption capacity of particle size fractions within natural aquatic sediments, but also to evaluate preferential distribution of steroid hormones between particle of various sizes within whole sediment. To do this, three types of sorption experiments were utilized; whole sediment sorption, sorption capacity where sediment was fractionated prior to equilibration with hormones, and preferential sorption where whole sediment was

equilibrated with hormone prior to rapid fractionation. The results will improve the understanding of steroid hormone distribution and bioavailability in aquatic systems.

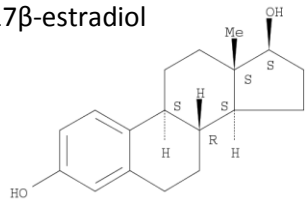
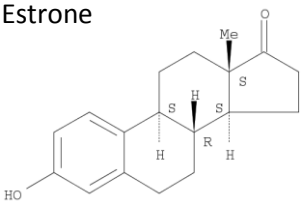
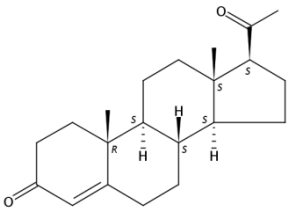
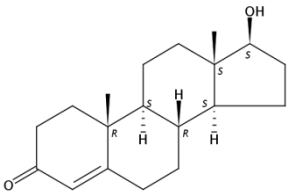
3.2 MATERIALS AND METHODS

Sediment preparation. Sand and silty loam sediments were collected as described previously (Chapter 2) Sediments were air dried before use. Properties of whole sediments and particle size fractions from each can be found in Table 2.1. Additional information about mineralogy was presented previously in Table 2.2. Each sediment was categorized according to particle size ranges as defined by USDA (Dane and Topp, 2002).

For sorption capacity experiments, sediment was fractionated using methods detailed in (Dane and Topp, 2002) utilizing both sieve and deposition methods. Briefly, sand was removed from the whole sediments using a wet sieve technique and was rinsed to remove any fine particles or debris that may have adhered to the sand particles. A repeated sedimentation procedure was used to isolate the remaining silt, clay, and colloid fractions. Approximately 100-200g of the remaining sediment was placed in a 1L beaker with water and sonicated to separate particles. This was allowed to sit undisturbed until particles became stratified. It was possible to estimate settling time needed for specific particles based on standard particle settling velocity equations and verifying the size of the particles in suspension using a zetasizer (NanoS90). The suspension containing the desired particle sizes was removed using a pipette and allowed to air dry before use. Due to a limited amount of fines in the sandy sediment, it was decided to assess the fines as a single fraction. 5 fractions consisting of sand (> 53µm), large silt (53-15 µm), small silt (15-2 µm) , clay (2-1 µm), and colloids(< 1µm) were evaluated in the silty loam sediment and 3 fractions consisting of sand-large (> 106µm), sand-small (106-53µm), and fines (<53µm) were evaluated in the sand. Physical properties associated with each

fraction can be seen in Table 2.1.

Table 3.1. Steroid hormone structure and properties.

Compound[a]	MW (g/mol)	Solubility (mg/L)[b]	Log K_{ow}
17β-estradiol 	272.4	3.0	3.10 – 4.01[c]
Estrone 	270.4	5.7	2.45 – 3.43[c]
Progesterone 	314.5	9.4	3.67-3.87[d]
Testosterone 	288.4	20	3.22[c]

[a] 4-^{14}C for all radiolabeled compounds. [b] Presented in SciFinder as calculated using ACD/Labs v11.02. [c] Cited by Lee et al. (2003). [d] Presented in ChemSpider as predicted by EPISuite.

Whole sediment sorption experiments. Equilibrium batch sorption experiments were run in triplicate using a combination of non-labeled (Sigma-Aldrich) and ^{14}C radiolabeled (American Radiolabeled Chemicals, St. Louis, MO) hormones, T, 17β-E2, E1, and Pr, with initial aqueous concentrations ranging between 5- 500 µg/L. Steroid hormone structures and select properties are shown in Table 3.1. Each hormone was delivered to experimental reactors using a stock solution of 250 mg/L hormone in ethanol. Solvent

was evaporated from each reactor under a steady stream of nitrogen before the addition of water and sediment to eliminate the possibility of solvent effects. Reactors consisted of approximately 1 g of sediment in 15 mL of deionized water containing 0.005 M CaCl and chemically sterilized with 200 mg/L NaN_3. Each reactor was capped with Teflon lined screw caps and rapidly mixed for 60 seconds and gently rotated in the dark for 24 hours. Equilibration periods were determined for T, E1, and 17β-E2 based on available literature data (Card et al., 2012; Lee et al., 2003; Qi et al., 2014)or determined experimentally for Pr (Figure 3.1). After equilibration, vials were centrifuged at 2000 rpm for 15 minutes. A 0.5 mL aliquot of supernatant from each reactor was assessed for radioactivity using a liquid scintillation counter, LSC, (Packard TRI-CARB 2500TR) to determine an aqueous concentration of hormone. The difference between the initial and final concentration in the aqueous phase was attributed to sorption of hormones to the sediment. This was verified by testing subsets of sediment directly using LSC. To do this, a representative aliquot of sediment ($\leq$0.1g) was placed in an LSC vial with 0.5 mL methanol and vortexed for 60s before measuring radioactivity. Testing soil directly allowed for recoveries of 94.2 $\pm$ 6.3% regardless of hormone or sorbent evaluated. Sorption isotherms were generated and fit using both Langmuir and Freudlich models. Negative controls containing solvent and sorbent, but no steroid hormones and positive controls containing solvent and hormone, but no sorbent were carried through the experiment to ensure there was no background contamination in the sediments and that hormone was not lost due to sorption to reactors.

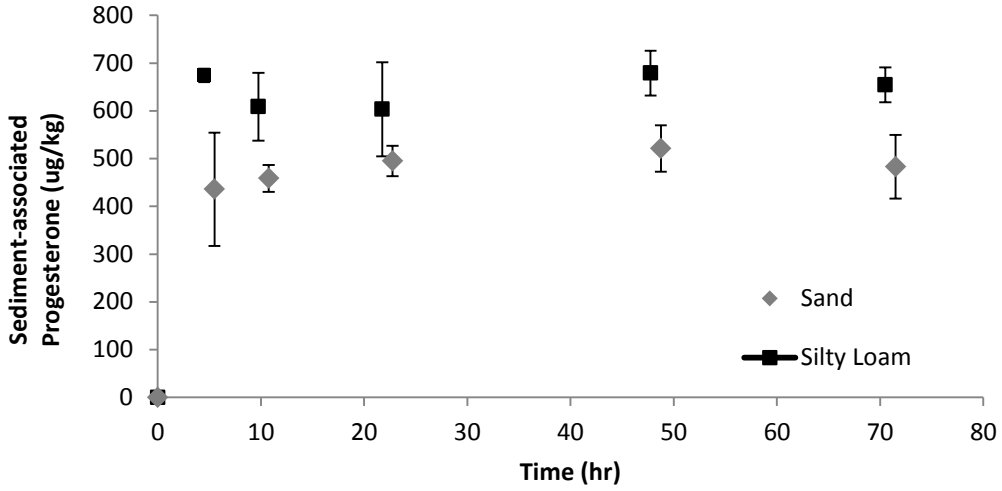


Figure 3.1. Progesterone time dependent sorption in sand and silty loam sediments at an initial aqueous concentration of 50 µg/L.

*Sorption capacity experiments.*Sorption capacity of particle size fractions was determined using the previously mentioned method with minor modifications. Reactor size was increased to 50 mL with approximately 0.8-1 g of each sediment fraction to ensure hormone could be detected in the aqueous phase for all particle size fractions evaluated using LSC. Additionally, the number of replicates was increased to 5 to allow for statistical analysis of data and only a single aqueous concentration of 500µg/L was used. Results were assessed for statistically significant differences using ANOVA followed by Tukey-Kramer multiple comparisons test in GraphPad Prism (6.01).

*Preferential sorption experiments.*To determine the effects of particle interactions within the whole sediments on hormone partitioning, a different set of sorption experiments was performed. Approximately 3.3 g of whole sediment was placed in 50 mL batch reactors with initial aqueous hormone concentrations ranging between 1 ng/L – 500 µg/L. After equilibration, the sediments were rapidly separated using deposition methods. Briefly, the

reactor was allowed to sit undisturbed for approximately 45s to allow the sand fraction to settle. The remaining suspension containing silt, clay, and colloids was removed. At this time, the samples derived from the sandy sediment were collected as a single fine group by centrifuging the suspension at 2000 rpm for 15 min. The silty loam sediment samples contained a greater abundance of fine particles and were fractionated further. It was possible to estimate theoretical settling times for silt particles as a function of gravity using accepted methods. A centrifuge was used to increase the force of gravity, G, by a factor of 8 and reduce the settling time to approximately 35 min as determined based on theoretical settling velocities and adjusted based on particle size measurements. The remaining suspension was removed and centrifuged again at 2000 rpm for 15 minutes to collect the clay and colloid fractions. Particle sizes were verified before testing subsets of each sediment fraction for radioactivity using LSC as previously described. Due to the rapid nature of the fractionation process, it was not possible to isolate the same number of fractions for each sediment as used in the sorption capacity experiments. However, it was possible to evaluate 3 fractions (sand, silt, and clay/colloids) in the silty loam sediment and 2 fractions (sand and silt/clay/colloids) in the sand.

3.3 RESULTS AND DISCUSSION

*Whole sediment sorption.*Linear sorption isotherms for 17β-E2, E1, T, and Pr were determined from triplicate measurements of equilibrium aqueous and sediment-associated concentrations for the two sediments evaluated (Figure 3.2). The data fit a linear sorption model with R^2 greater than 0.96 in the silty loam sediment and greater than 0.89 in the sand (Table 3.2) indicating linear partitioning was dominant over the concentration ranges evaluated. Non-linear model parameters are provided in Table 3.3 with Freudlich exponents greater than 0.86 indicating near linear model fit. The use of linear sorption models is consistent with results from Card et al. (2012), which determined linear sorption model fit sorption isotherm data in agricultural soils over several orders of magnitude. Other previous studies have fit steroid sorption with linear or near linear models (Gineys et al., 2012; Casey et al., 2005; Casey et al., 2004; Lee et al., 2003; Sangsupan et al., 2006), while others report improved model fits for non-linear isotherms models (Gineys et al., 2012; Lee et al., 2003). Measured values of K_d in the silty loam sediment for 17β-E2, E1, and T were well within the range of values previously reported in other studies (Casey et al., 2005; Casey et al., 2004; Lee et al., 2003). However, average K_d values

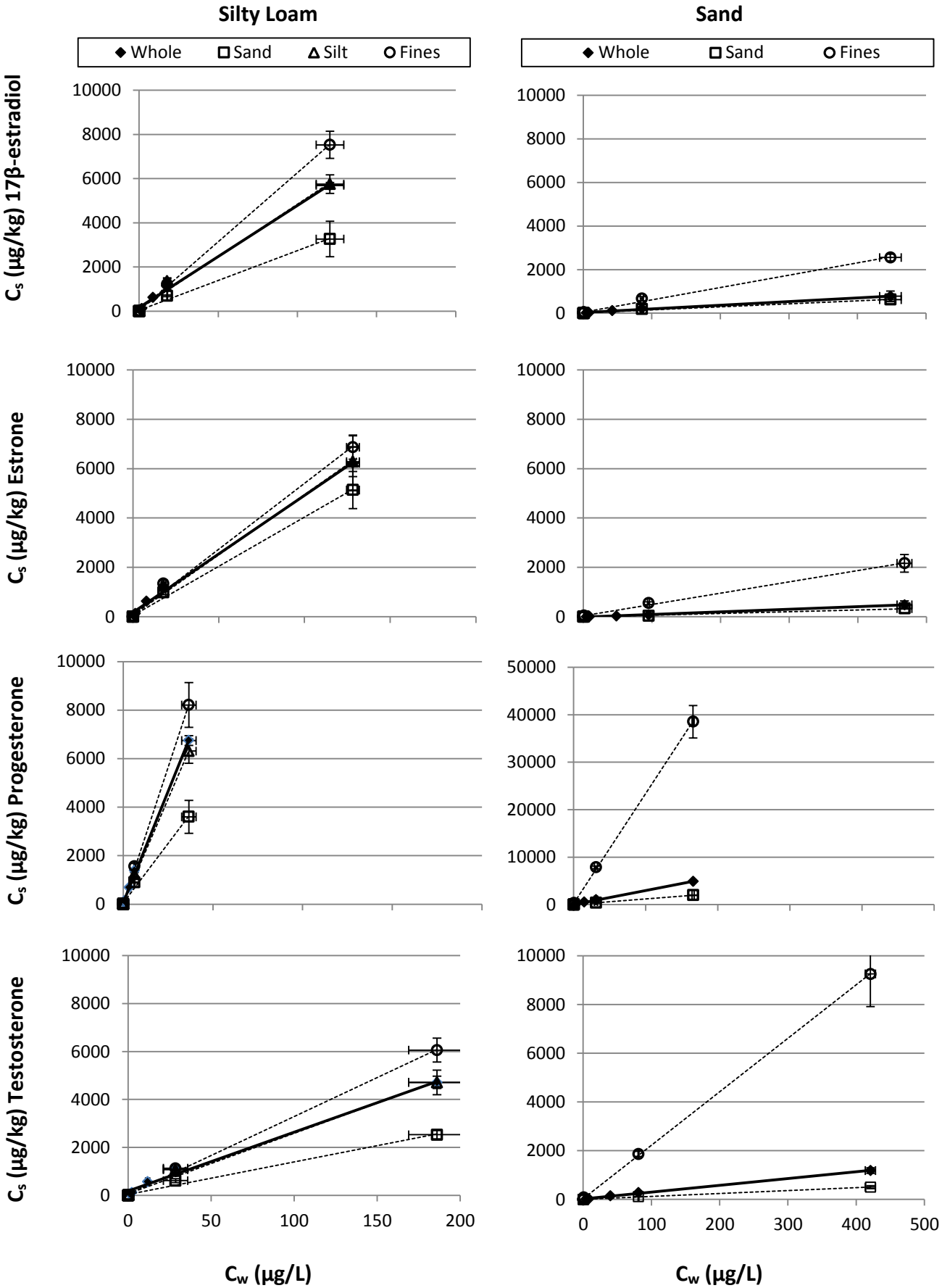


Figure 3.2. Linear sorption isotherms for whole sediments (solid lines) and sediment fractions (dashed lines) obtained during preferential sorption experiments.

measured for 17β-E2 (2.54 L/Kg) and E1 (1.10 L/Kg) in sand were lower than previously reported values. With the exception of E1 in the sandy sediment, all log K_{oc} values were consistent with those reported previously (Table 3.2). Differences in hormone sorption to the two sediments may be related to differences in minerology determined for the two sediments. The silty loam sediment consists of 50% mixed layer illite/smectite (Table 2.2) while the sandy sediment is predominantly illite. In this study, higher sorption was observed to expansive clays, which is comparable with previous studies which determined higher degree of sorption of estrogens to montmorillonite when compared with non-expandable clays such as kaolinite (Shareef et al., 2006). Because the pH of both sediments at7.4 was below the pK_a of the steroids, sorption is likely due to hydrophobic interactions between the compounds and sediment organic matter.

The lower log K_{oc} measured for E1 (2.60) in the sandy sediment in this study as compared to reported literature values may be due in part to slight differences in methodology including the range of initial aqueous concentrations utilized. Additionally, the sandy sediment used in this study contained lower quantities of organic carbon than the soils used in prior studies reported in Table 3.2. It is also important to note the possibility of abiotic estrogen transformation processes may occur and would not be accounted for in the present study. Oxidation of estrogens has been observed in soil slurries containing manganese (Sheng et al., 2009); however XRD analysis conducted on the sediments evaluated in this study provides evidence of low metal content. In addition, in water-sediment systems similar to those evaluated in this study, abiotic loss of estrogens, 17β-E2, E1, and EE2 have been shown to be minimal within the first 24 hours

but may show a marked increase by 7 days (Mashtare et al., 2013; Sarmah and Northcott, 2008; Ying and Kookana, 2005).

For both soils tested, Pr had a higher affinity for the solid phase than the other hormones evaluated with average K_d values of 33.3 L/kg in the sand and 207.2 L/kg in the silty loam sediments (Table 3.2) as well a higher log K_{oc} values for Pr when compared to the other hormones evaluated. Log K_{oc} values for Pr determined in this study are comparable to log K_{om} determined by Neale et al. (2009) in a study evaluating Pr sorption to 3 types of organic matter at pH values comparable with this study.

Table 3.2. Results of whole sediment sorption using linear isotherms.

					Reported Literature Values	
Compound	**Sediment Type**	**K_d (L/kg)**	**R^2**	**Log K_{oc}**	**K_d (L/kg)**	**Log K_{oc}**
17β-estradiol	Silty Loam	71.9	0.99	3.45	3.56-89.47	3.24 ± 0.39[a,b]
	Sand	2.54	0.89	3.04		
Estrone	Silty Loam	75.1	0.96	3.47	3.40-108	3.32 ± 0.21[a,b,c]
	Sand	1.10	0.89	2.60		
Progesterone	Silty Loam	207.2	0.98	3.91		4.34 ± 0.8[d]
	Sand	33.3	0.99	4.16		
Testosterone	Silty Loam	43.3	0.97	3.23	4.57-42.7	3.17 ± 0.26[a,e]
	Sand	3.45	0.99	3.18		

[a]Lee et al. (2003) [b]Casey et al. (2005) [c] Ying et al.(2005) [d]From 3 types of organic matter tested (pH 7-9) by Neale et al. (2009) [e]Sanguspan et al. (2006).

Table 3.3. Non-linear sorption isotherm parameters for whole sediments using Langmuir and Freudlich models.

		Langmuir Model			Freudlich		
Compound	**Sediment Type**	q_m (µg/g)	K_L (mL/g)	R^2	K_f ($\mu g^{1-n} L^n/kg$)	n_f	R^2
17β-estradiol	Silty Loam	1.43	70.00	0.95	87.10	0.88	0.98
	Sand	5.00	0.50	0.86	3.28	0.91	0.96
Estrone	Silty Loam	5.00	18.52	0.99	95.13	0.87	0.99
	Sand	0.67	1.18	0.80	1.19	0.99	0.90
Progesterone	Silty Loam	5.00	42.55	0.98	204.83	0.99	0.99
	Sand	14.29	2.44	0.99	36.02	0.97	0.99
Testosterone	Silty Loam	3.33	15.54	0.92	57.33	0.86	0.98
	Sand	1.11	3.36	0.96	4.01	0.95	0.99

*Sorption capacity as a function of particle sizes.*Particle size fractions obtained from the silty loam and sandy sediments were equilibrated with 17β-E2, E1, Pr, or T at a single initial aqueous concentration (500 µg/L) with differences in sorbed concentration assessed for statistical significance ($p < 0.05$) for each particle size fraction based on source sediment and hormone (Figure 3.3). Additionally, estimated K_d values from each fraction and steroid were used to determine relationships between the K_d values and physical charateristics of each particle size fraction (Table 3.4). While complete sorption isotherms were not measured, a previous study evaluating sorption of T and EE2 to sediment particle fractions yielded isotherms were described by linear or near linear models (Qi et al., 2014; Sun et al., 2012).

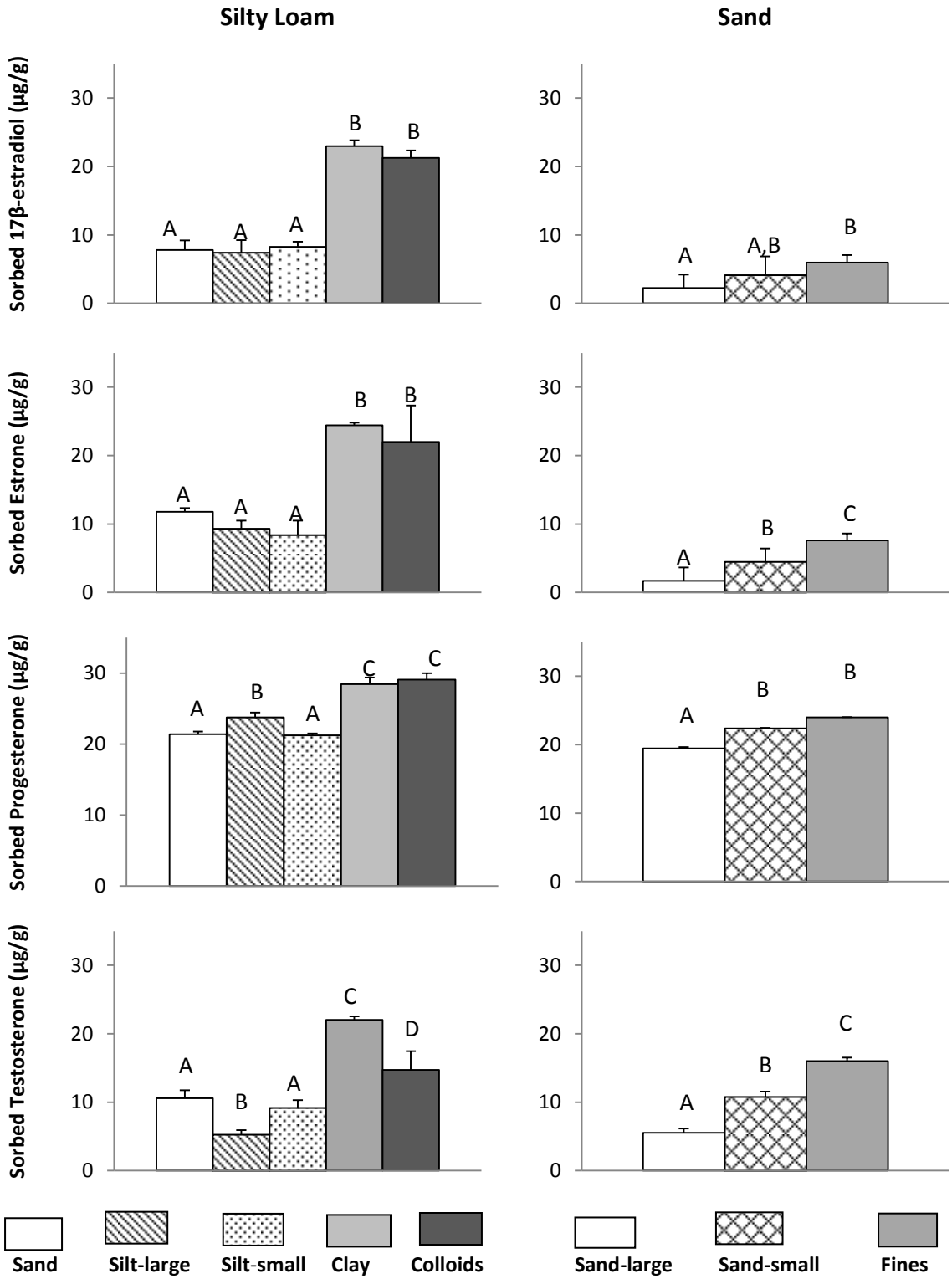


Figure 3.3.Sorption capacity of four steroid hormones to different particle size fractions of a silty loam (left column) and a sandy sediment (right column) reported as average ± sd. Letters denote statistically significant relationships between groups for an indicated steroid.

Table 3.4. Summary of correlation coefficients from linear regressions between K_d (L/kg) and physical characteristics of sediment fractions used in sorption capacity experiments.

	Silty Loam			Sand		
	CEC[A]	% OC[B]	SA[C]	CEC	% OC	SA
17β-estradiol	0.89	0.89	0.93	0.95	0.98	0.99
Estrone	0.84	0.93	0.99	0.93	0.99	0.99
Progesterone	0.88	0.67	0.85	0.99	0.9	0.93
Testosterone	0.54	0.98	0.71	0.94	0.98	0.99
CEC vs %OC	0.65			0.86		
%OC vs SA	0.8			0.99		
SA vs CEC	0.95			0.9		

[A] Cation Exchange Capacity [B] Percent organic carbon [C] Surface Area

All hormones evaluated exhibit significantly higher sorption capacity in the clay and collloid fractions of the silty loam sediment when compared to the sand and silt fractions (Figure 3.3). Hormone sorption to colloids is not significantly different from sorption capacity of the clay fraction for 17β-E2, E1, and Pr. However, in the case of T, colloids exhibited a lower sorption capacity than that of the clay fraction. This behavior may be due to the sensitivity of T to the percentage of OC found in each fraction as this hormone exhibited weaker correlation with CEC and surface area (Table 3.4) for the sediment used in this study. Comparing log K_{oc} values across the particle size fractions for T sorption showed no significant differences ($p > 0.05$) between the groups except for the clay and colloid fractions (Figure 3.4). This indicates OC is the primary sorption domain and is consistent with other studies (Lee et al., 2003; Qi et al., 2014). The studies evaluating T sorption in whole sediments and in partical size fractions have shown a strong correlation between both OC and surface area (Lee et al., 2003; Qi et al., 2014). However, in these studies both OC and surface area increase as soil texture becomes finer

or as particle size decreases and it is not possible to look at the two physical characteristics separately. In this study surface area and CEC increased with decreasing particle size in fractions of the silty loam sediment, while, the fraction of OC in the clay fraction (6.39) is greater than in the colloids (4.17) (Table 2.1). Sun et al. (2012) saw a similar lack of correlation between surface area and OC content of particle size fractions used in sorption experiments and determined that BPA and phenanthrene sorption was strongly correlated with OC while EE2 sorption was strongly correlated to OC in a sediment but more strongly correlated to surface area in soil fractions. The estrogens, 17β-E2 and E1, sorbed at comparable levels for both the sand and silt fractions as indicated by lack of statistically significant differences in these groups ($p>0.05$) for each estrogen (Figure 3.3). In contrast, Pr and T, behaved differently in the sand and silt fractions with Pr exhibiting a significantly greater sorbed concentration in the large silt fraction (23.8 ± 0.7 µg/g) as compared to the sand and small silt fractions (21.2 ± 0.3 µg/g) and T exhibiting significantly lower sorption in this fraction (5.25 ± 0.7 µg/g compared to 10.6 ± 1.16 µg/g). This may be due to the lower values of CEC, OC, and surface area measured in the larger silt fraction compared to the sand and smaller silt fractions (Table 2.1) that is compounded by the strong correlation between T and the percentage of OC and a weaker correlation between Pr and the percentage of OC (Table 3.4). Several studies have shown the importance of hydrogen bonding in steroid-organic matter interactions (Yamamoto et al., 2003; Yu and Huang, 2005) and the degree of sorption of hormones is also dependent on the characteristics of the organic matter present and the presence and location of hydroxyl and ketone functional groups within each hormone (Neale et al., 2009). Ketone fuctional groups are strong hydrogen aceptors

and are present on both T and Pr in the C-3 position with an additional present in C-20 in Pr. Although a correlation between CEC and hormone sorption was observed, this is likely due to the fact that CEC and surface area were highly correlated for the silty loam sediment.

Relationships between CEC and hormone sorption have been documented in previous studies (Casey et al., 2003; Khan et al., 2009). The correlation between hormone sorption and surface area has been observed previously, with higher degree of hormone sorption observed for montmorillonite clays with potential for intercalation (Shareef et al., 2006). Scherr et al. (2009) found that sorption of estrogens to agricultural soils was limited by the number of sorption sites available, and that these sorption sites were allocated primarily within the organic matter domain of the soils. This finding may also explain the correlations between hormone sorption, OC, and SA.

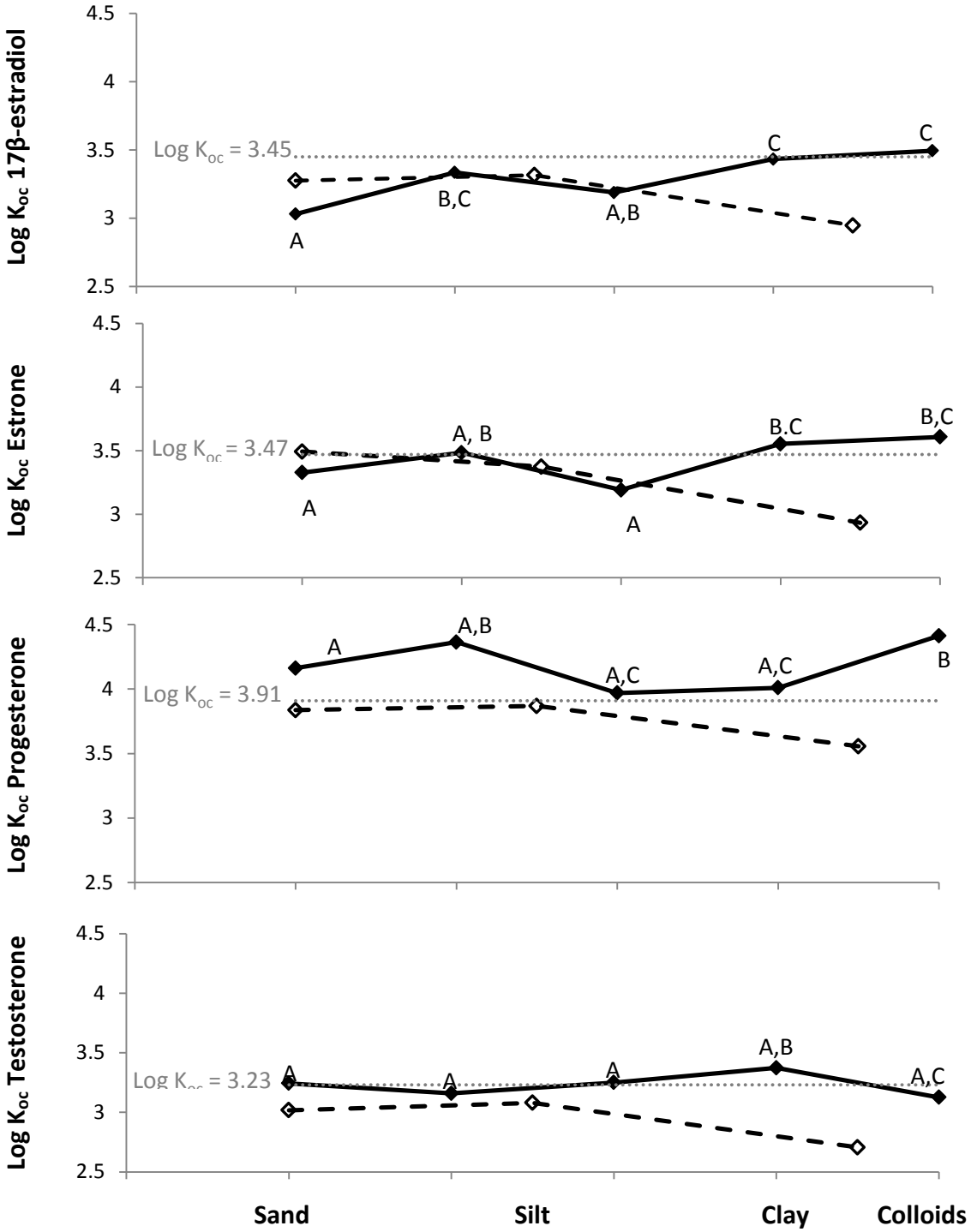


Figure 3.4. Organic carbon normalized sorption distribution coefficients (Log K_{oc}) for whole sediment (gray) and particle size fractions from a silty loam sediment. Solid lines and markers represent results from sorption capacity experiments with statistically significant relationships denoted. Dashed lines represent results obtained from preferential sorption experiments.

Since the sandy sediment did not include a diversity of particle sizes, it was not possible to evaluate as many fractions as in the silty loam sediment (Figure 3.3). However, there was a greater affinity for the finer particle sizes for all hormones evaluated due to increased CEC, percentage of OC, and surface area in this particle fraction and to the strong correlation between these physical characteristics in this particular sediment. Additionally, there was no significant differences ($p > 0.05$) in Log K_{oc} values between different particles size fraction for each hormone evaluated (Figure 3.5).

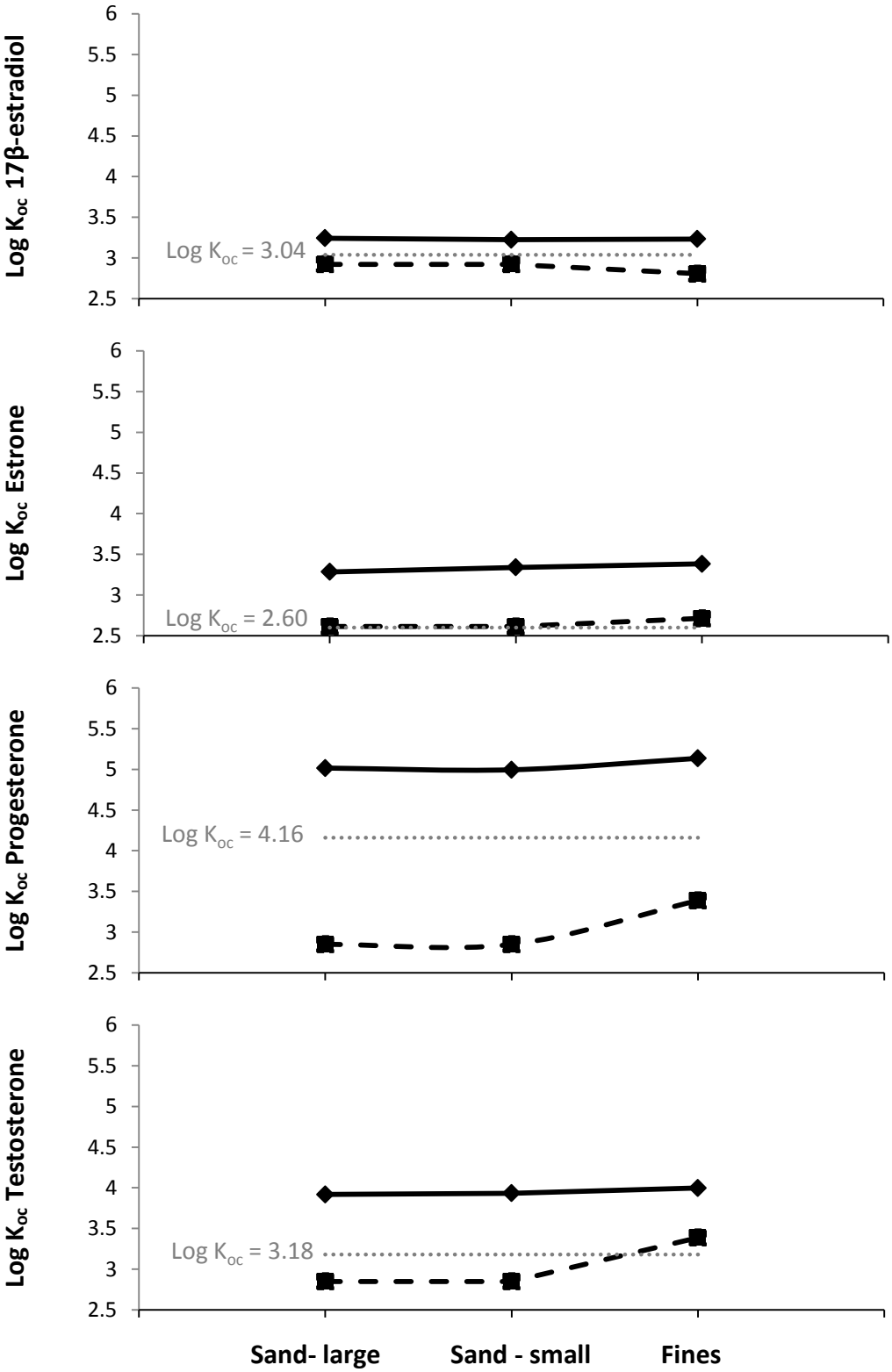


Figure 3.5. Organic carbon normalized sorption distribution coefficients (Log K_{oc}) for whole sediment (gray) and particle size fractions from a sandy sediment. Solid lines and markers represent results from sorption capacity experiments. Dashed lines represent results obtained from preferential sorption experiments.

Preferential sorption of steroids to particle size fractions of a whole sediment.When sediments were fractionated after equilibrium sorption was achieved, hormones show a marked affinity for smaller particle size fractions in both sediments (Figure 3.2) and are well described with a linear model (Table 3.5) with $R^2 \geq 0.98$ for all particle size fractions and steroids evaluated with the exception of 17β-E2 in the sand fraction of the silty loam sediment ($R^2 = 0.95$) and E1 in the fine fraction of the sand ($R^2 = 0.90$). Whole sediment sorption aligns most closely with the sorptive behavior of the dominant fraction (by weight) found in each sediment. This is intuitive as the dominant fraction in the silty loam (silt) was 60% (by weight) of the whole sediment and the sandy sediment is 92% sand. This trend continues when evaluating log K_{oc} as seen in the particle fractions from the silty loam sediment (dashed lines in Figure 3.4). However, the relationship between log K_{oc} and sandy sediment is not as closely aligned with the sand fraction (Figure 3.5). The particle fractions from the silty loam sediment also exhibit a reduction in log K_{oc} in the fine fraction likely due to an increase in organic carbon as particle size decreases (Figure 3.4).

Table 3.5. Linear sorption isotherm parameters for preferential sorption experiments where sediment fractions were rapidly separated after whole sediment equilibration.

	Silty Loam						**Sand**			
Fraction	Sand		Silt		Fines		Sand		Fines	
Compound	Kd (L/kg)	R^2	Kd (L/kg)	R^2	Kd (L/kg)	R^2	Kd (L/kg)	R^2	Kd (L/kg)	R^2
17β-estradiol	22.01	0.95	38.93	0.99	50.25	0.99	1.00	0.98	4.07	1.00
Estrone	30.29	0.98	41.17	0.99	44.98	0.99	0.75	1.00	5.15	0.90
Progesterone	99.98	0.99	173.51	0.99	255.42	0.99	12.00	1.00	233.53	0.99
Testosterone	6.34	0.99	11.77	0.99	14.99	0.99	1.21	0.99	22.04	0.99

Figure 3.6 presents the distribution of total sediment-associated hormone as a function of particle size. In the silty loam sediment (left column Figure 3.6), the majority of the sorbed hormone is found associated with clay and colloids at low initial aqueous

concentrations (1 ng/L). This is approximately 80% of E1 and over 50% for 17β-E2, T, and Pr in a fraction that amounts to 24% of the whole sediment by weight. The sand fraction (approximately 16% by weight) is consistent across the range of initial aqueous concentrations at approximately 8-12%. As the initial aqueous concentration increased for each hormone, percent of sorbed hormone begins to follow a trend similar to the percent soil texture. In the sandy sediment (right column in Figure 3.6), the amount of sorbed 17β-E2 follows a similar trend. At lower initial aqueous concentrations, up to approximately 93% of sediment-associated E2 can be found in the finer particles (8% by weight). E1 and T vary over the range of aqueous concentrations and Pr is fairly consistent with approximately 63-72% of sorbed Pr in the fine particles.

Preferential hormone distribution in smaller particles may be due in part to a two-phase sorption process that has been observed previously for clay minerals. The first phase involves rapid sorption with one study finding 50 – 80% of 17β-E2, E1, and EE2 associated with smaller particles (< 1μm) within 30 min of sorption(Duong et al., 2010b). During the second phase, hormone sorption slows indicating diffusion into interlayers or micropores within the sediment particles. The strong correlation between hormone sorption and SA and/or OC (Table 3.4) found in this study suggests the importance of adsorption processes across the particle size fractions evaluated in each sediment. The higher measured SA and OC in the finer particles may limit sorption of hormone to larger particle fractions within the whole sediment initially.

Preferential sorption of hormone to smaller particle size fractions is likely more pronounced at lower concentrations due to the limited amount of hormone in the system. However, the small particle fractions represent a small portion (24% in the silty loam

sediment and 8% in the sand) of the overall sediment, and as the concentration of hormone increases, increasing amounts of hormone are sorbed to the larger size fractions. Additionally, due to the limited amount of fine particles (8%) present in the sand, the effects of preferential sorption are not as consistent or clear. In environmental systems with low steroid: sediment ratios, the steroid will preferentially associate with the small particles, but when the steroid:sediment ratio is higher, steroid will be found across various particle sizes.

*Environmental implications in aquatic systems.*The effects of increased sorption capacity and preferential distribution in smaller particle size fractions may have ramifications on environmental systems. While the mechanism of steroid hormone exposure via sediments to aquatic organisms is not well understood, there is a growing body of evidence to suggest that sediment can act as source of biologically active compounds (Jeffries et al., 2011; Jessick et al., 2014; Sangster et al., 2014; Sellin et al., 2010) and that association with finer particles may enhance steroid hormone bioavailability in fish (Duong et al., 2009). The sediment-water interface found in benthic environments is subject to near constant mixing during normal stream flow, preciptitation events, and pertubation of the stream bed by aquatic organisms. This study suggests particle interactions may cause a prefferential distribution of hormones within fine partcle size fractions of whole sediments with up to 93% of sediment associated hormone found in the fine fraction at low aqueous concentrations (1 ng/L). These smaller particles have a greater tendency to be suspended in and move with the water column. Therefore, compounds associated with these particles are more likely to travel greater distances. Additionally, aquatic organisms may have more contact, through ingestion or respiration across gills, with smaller particles; thus, increasing their exposure to hormones.

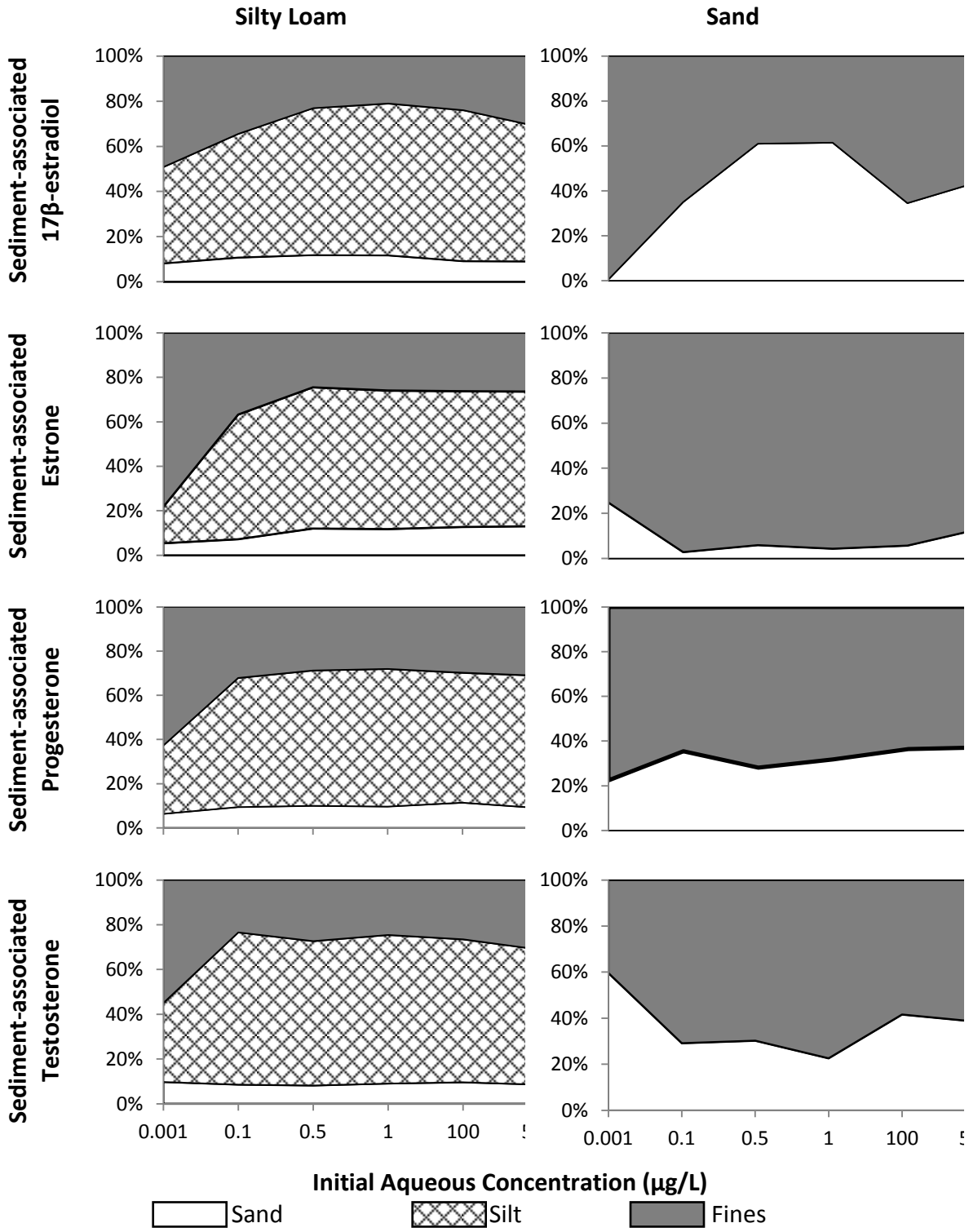


Figure 3.6. Sorbed steroid hormone distribution expressed as percent of total sediment-associated steroid within three fractions of a silty loam (left column) and two fractions of a sandy (right column) sediment based on initial aqueous concentration. Fines are defined as clay and colloids for the silty loam sediment and as silt, clay, and colloids in the sandy sediment.

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CHAPTER 4: BIOAVAILABILITY AND FATE OF SEDIMENT-ASSOCIATED PROGESTERONE IN AQUATIC SYSTEMS

This chapter presents data published in the following peer-reviewed publication:

Sangster, JL; Ali, JM; Snow,DD; Kolok, AS; Bartelt-Hunt, SL. Bioavailability and fate of sediment-associated progesterone in aquatic systems. *Environ. Sci. Technol.* **2016,** 50 (7): 4027-4036.

4.1 INTRODUCTION

There is growing concern about potential endocrine disrupting effects stemming from the occurrence of progesterone (Pr) in aquatic systems. Several recent studies have suggested P4 may be responsible for the increased presence of androgens in soil and water adjacent to agricultural areas (Gall et al., 2011; Jenkins et al., 2003; Mansell et al., 2011) and may explain the masculinization of female fish downstream of paper mills, an industry known to release significant amounts of plant-derived progesterone into aquatic systems (Jenkins et al., 2003). A recent review reported average Pr concentrations below 5 ng/L in surface waters receiving wastewater treatment plant effluent, although concentrations as high as 199 ng/L were also reported (Kumar et al., 2015). Studies evaluating paper mill effluents detected aqueous Pr in the low µg/L range (Jenkins et al., 2003) and studies of runoff from beef cattle feedlots found aqueous Pr at concentrations as high as 1070 ng/L (Bartelt-Hunt et al., 2012; Mansell et al., 2011) with average feedlot soil concentrations of 148 ng/g (Mansell et al., 2011). Exposure of adult female fathead minnows (*Pimephales promelas*) to aqueous Pr at concentrations as low as 10 ng/L resulted in reduced reproductive success and reduced expression of vitellogenin (Vtg), an egg yolk precursor protein (DeQuattro et al., 2012). Additionally, studies have documented endocrine effects in zebra fish (*Danio rerio*) at aqueous Pr concentrations as

low as 3.5 ng/L in adult females (Zucchi et al., 2013) and after embryonic exposure to concentrations as low as 2 ng/L(Zucchi et al., 2012).

Although Pr has been shown to be released in runoff from areas treated with manure from animal production facilities (Bartelt-Hunt et al., 2012; Gall et al., 2011; Mansell et al., 2011), there are limited studies evaluating Pr occurrence in surface waters adjacent to agricultural areas. This may be due in part to the spatially and temporally diffuse nature of agricultural runoff, which results in episodic loading of contaminants during periods of snowmelt, precipitation, and irrigation (Gall et al., 2011; Mansell et al., 2011). In aquatic systems susceptible to episodic loading of steroid hormones, there is growing evidence of the importance of sediment interactions on both contaminant fate (Khan and Lee, 2010; Mashtare et al., 2013; Sangster et al., 2015; Stumpe and Marschner, 2010) and the subsequent bioavailability to aquatic organisms (Duong et al., 2009; Jessick et al., 2014; Sangster et al., 2014; Sellin et al., 2009; Sellin et al., 2010). In a previous study, we determined fathead minnows exposed to sediment-associated trenbolone exhibited significant differences in Vtg expression that could not be explained solely by measured concentrations of aqueous steroids (Sangster et al., 2014). More recently, Zhang et al. (2015) evaluated agrichemical fate and bioavailability in field-based mesocosms within an agriculturally-influenced watershed and determined that exposure to river sediment was necessary for the observed biologic response in fathead minnows.

There is a growing body of work indicating sediment composition can affect the bioavailability of steroids in aquatic systems (Duong et al., 2009; Sangster et al., 2014; Zhang et al., 2015) by influencing compound fate as it relates to distribution (Qi et al.,

2014; Sangster et al., 2015; Sun et al., 2012) and degradation (Khan et al., 2008; Khan and Lee, 2010; Mashtare et al., 2013) in sediment-water systems. It is unclear if biological effects stemming from sediment-associated steroids are due to desorption from the sediments and/or by direct contact between fish and sediment-associated steroid. Some studies have shown limited detection of aqueous phase steroid (Sangster et al., 2014; Sellin Jeffries et al., 2011; Sellin et al., 2010) suggesting direct contact may at least be partially responsible for the observed biologic effects while one study found that steroid interactions with finer particle size fractions actually enhanced biological effects of the steroid (Duong et al., 2009). Recently, Jessick et al.(2014) attempted to evaluate the mechanism by which sediment-associated trenbolone (Tb) is bioavailable to female fathead minnows. It was determined that direct contact with whole sediments is not necessary for defeminization of fish to occur. It was not possible to determine conclusively if effects seen in the fish were caused by Tb associated with a small quantity of fine particles in the water column or by free steroid that had desorbed from sediments. A few studies have shown increased sorption capacity and preferential sorption of steroids to finer particle size fractions within whole sediments with a higher degree of desorption from coarser fractions which may help to explain the effects observed in the fish. Additionally, sediment texture may affect the degradation rates and metabolites formed during microbial degradation of the parent compound (Khan et al., 2008; Khan and Lee, 2010; Mashtare et al., 2013) which will affect biological response in aquatic vertebrates, such as fish. Several studies have evaluated aqueous Pr degradation by pure species of bacteria including *Aspergillus, Anthrobacter, Chlorella, Penicillium, Bacillus,* and *Streptomyces* (Bartmanska et al., 2005; Hunter and Carragher, 2003; Hunter et al.,

2009; Mostafa and Zohri, 2000; Yildirim et al., 2011) with intermediate products reported, including the androgens testosterone acetate, testosterone (T), hydroxytestosterone, androstenedione (AD), and testolactone, an anti-estrogen. To date, the transformation and bioavailability of Pr have not been evaluated in more complex sediment-water systems.

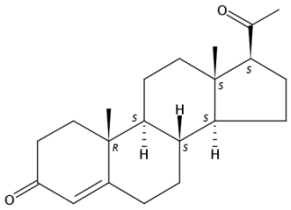
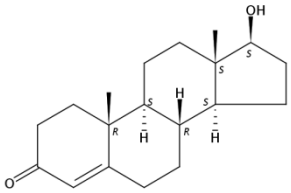
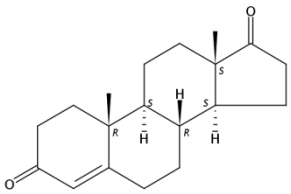
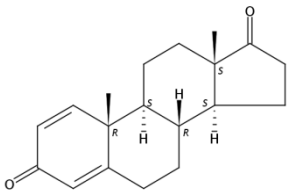
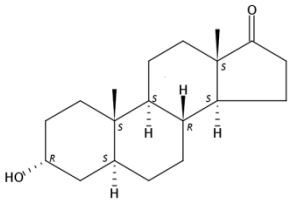
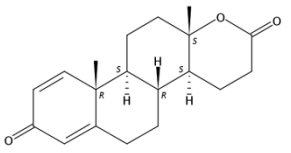
The present study was designed to evaluate how differences in sediment composition affect Pr fate as well as the biologic response of fathead minnows to Pr under conditions that are consistent with agriculturally-impacted aquatic systems. Two natural aquatic sediments with marked differences in texture and organic carbon content were used. Biological effects stemming from exposure to sediment-associated Pr were determined by evaluating differences in hepatic expression of Vtg, androgen receptor (AR), and estrogen receptor alpha (ERα) in an environmental sentinel species, the fathead minnow. Compound fate was determined by measuring aqueous and sediment-associated steroid hormones in both the fish exposure aquaria and in controlled microcosms. Results of the biological analysis were compared with analytical chemistry data obtained from controlled microcosms and periodic grab samples of sediment and water from exposure aquaria to correlate contaminant fate with bioavailability in the target organism.

4.2 MATERIALS AND METHODS

Chemicals and reagents. All reagents used in this project were purchased from Fisher Scientific and used the highest purity available (Optima, Thermofisher Scientific, St. Louis, MO). Pure steroids (Table 4.1), including progesterone, testosterone, 4-androstenedione, androsterone, androstanedienedione, and testolactone were purchased from Sigma-Aldrich (St. Louis, MO) or Acros Chemicals with ^{14}C radiolabeled progesterone from American Radiolabeled Chemicals (St. Louis, MO). Internal standards included testosterone-d3, obtained from Sigma Aldrich (St. Louis, MO), and $^{13}C_6$-estradiol purchased from Cambridge Isotopes (Andover, MA).

Sediment preparation. Two natural aquatic sediments (as described in Chapter 2) were used in this study to represent differences in physical properties; a silty loam collected from Plum Creek in Seward, Nebraska and a sand from the Elkhorn River near Winslow, Nebraska. To ensure natural microbial activity would be present, sediment was collected within 36 hr of the beginning of experiments and kept in a cool dark location until use. Select properties of each sediment are presented (Table 2.1) with additional information about specific mineralogy (Table 2.2).

Table 4.1. Properties of steroid hormones.

Compound	MW (g/mol)	Solubility (mg/L)[A]	Log K_{oc}
Progesterone 	314.5	9.4	4.16, 3.91[B]
Testosterone 	288.4	20	3.18, 3.23[B]
4-Androstenedione 	286.4	34	2.85[A]
1,4-Androstanedienedione 	284.4	34	2.80[A]
Androsterone 	290.4	7.3	3.52[A]
Testolactone 	300.4	28	2.59[A]

[A] Presented in SciFinder as calculated using ACD/Labs v11.02. [B] Determined using sediments from this study for sand and silty loam respectively(Sangster et al., 2015).

Fish exposure experiments. Laboratory experiments were conducted in 45-L glass aquaria using sexually mature fathead minnows from an on-site culture using previously described protocols (Jessick et al., 2014; Sangster et al., 2014). Nine groups consisting of 40 fish each (20 male and 20 female) were used with 4 groups exposed to each sediment (sand and silty loam) at sediment concentrations of 0, 5, 50, and 500 ng/g (dry weight) Pr. The final group consisted of fish unexposed to sediment or hormone (i.e. lab water only) to ensure that any observed change in gene expression could not be attributed to pre-existing contaminants in the sediments or to solvents used in hormone stock solutions (data not shown). In the experimental tanks using sediment, a layer of sediment approximately 6 cm deep (8 kg dry weight) was pre-equilibrated with Pr and was not replenished during the experiment. All aquaria contained 45 L aerated and dechlorinated tap water at 26±0.2°C with a photo period of 16:8 h light:dark. Static renewal of the water was performed at a rate of 0.3 d^{-1}. Fish were fed twice daily with a commercially available food, Tetramin (Melle, Germany), and frozen *Artemia salina*.

To simulate progesterone-contaminated sediment, a solution containing Pr dissolved in methanol and deionized water was added to six of the eight 45 L aquaria containing sediment. Each solution and sediment was combined in separate aquaria and mixed thoroughly every few hours over a 12-hour period to ensure a homogeneous substrate. The final methanol concentration was kept to a minimum (<0.1 µL/L) to avoid solvent effects in the fish (Sangster et al., 2014). After mixing, all tanks were allowed to further equilibrate with water for 12 hours before the addition of fish. Pr has been shown to reach equilibrium within 24 hrs for these sediments (Sangster et al., 2015).

Target concentrations of 5, 50, 500 ng/g were chosen based on previously

published research detailing Pr occurrence in agriculturally impacted waterways. Sediment concentrations of Pr have been detected at or below 50 ng/g in aquatic sediments (Jenkins et al., 2003) and represent known environmentally relevant concentrations; however, many studies evaluating occurrence of steroids in the environment assess only aqueous steroid and do not measure steroid in sediments. Therefore, the 500 ng/g exposure groups serve as a possible worst case scenario of environmental contamination while allowing for detection of both parent steroid and metabolites in both the aqueous and sediment phases using analytical chemistry techniques.

Sediment and water samples were collected from each aquaria after 24 hr of equilibration but immediately before the introduction of fish (e.g. 0 d); and daily for the duration of the exposure period. At each sampling event, a 250 mL aqueous grab sample was collected and stored in an amber glass bottle at -20°C until analysis. A composite sediment sample was obtained by collecting three to four 10 g randomly-selected sediment subsamples within each aquaria and stored -20°C until analysis.

Biological analysis. Since steroid hormones are subject to biotransformation and excretion within organisms, the effective bioavailability of compounds was inferred based on molecular changes in estrogen responsive, anti-estrogen responsive, and androgen responsive genes. To evaluate bioavailability, ten male and ten female fish were randomly collected from each aquaria at 7 d with the remaining harvested at 14 d. After collection, fish were euthanized and weighed. Livers and gonads were collected from each individual and weighed. These were then flash frozen in liquid nitrogen and stored at -80°C until analysis. Hepatosomatic index (HSI) and gonadosomatic index (GSI) were

calculated for each fish as the mass of the tissue divided by the total mass of the fish multiplied by 100 (Table 4.2).

Table 4.2. Morphometric data for fathead minnows exposed to sediment-associated progesterone.

	Exposure Concentration (ng/g)	Females		Males	
		HSI (%)	GSI (%)	HSI (%)	GSI (%)
7 Day					
Sand	0	4.6 ± 2.1	13.5 ± 1.9	3.6 ± 1.0	2.0 ± 0.6
	5	3.9 ± 1.5	15.4 ± 3.6	3.0 ± 0.6	2.0 ± 0.7
	50	2.6 ± 0.8	15.3 ± 3.9	3.4 ± 1.1	1.8 ± 0.4
	500	3.7 ± 1.1	11.8 ± 2.6	2.3 ± 1.0	1.7 ± 0.6
Silty Loam	0	3.3 ± 1.2	13.5 ± 3.2	2.7 ± 0.6	2.3 ± 0.5
	5	3.1 ± 1.2	13.7 ± 4.7	2.9 ± 0.7	2.2 ± 0.4
	50	3.3 ± 0.9	12.6 ± 2.2	2.6 ± 0.5	2.0 ± 0.3
	500	3.6 ± 1.5	12.7 ± 3.3	2.6 ± 0.3	3.0 ± 0.9
14 Day					
Sand	0	5.5 ± 1.1	14.8 ± 3.2	5.1 ± 4.4	2.8 ± 1.5
	5	5.4 ± 2	12.5 ± 2	2.7 ± 0.8	3.2 ± 1.4
	50	4.9 ± 1.7	13.4 ± 3.4	3.8 ± 0.8	1.9 ± 0.6
	500	5.1 ± 1.8	13.6 ± 4.5	3.3 ± 0.8	1.9 ± 0.7
Silty Loam	0	4.4 ± 1.5	13.7 ± 2.6	3.7 ± 1.6	1.9 ± 0.3
	5	3.1 ± 2.3	11.1 ± 3.4	3.4 ± 0.6	2.2 ± 0.8
	50	3.8 ± 1.1	14.3 ± 4.5	2.9 ± 0.8	1.9 ± 0.6
	500	3.4 ± 0.6	18.9 ± 4.8	3.5 ± 0.5	2.1 ± 0.5

Hepatic Vtg, AR, and ER-α mRNA expression was evaluated using ribosomal L8 as a normalization standard and expressed in relative terms as outlined in Schmittgen and Livak (2008). Briefly, RNA was extracted from liver tissue using the SV Total RNA Isolation System (Promega) in accordance to the manufacturer's recommendations (Promega, Madison, WI, USA). Extracted RNA was then converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). All RT-qPCRs were performed in duplicate using iQ SYBRGreen Supermix (Bio-Rad) with primers obtained from EurofinsGenomics (Huntsville, AL, USA). Amplification efficiencies were determined using a standard

curve prepared from a serially diluted pool of cDNA and were between 85.2 and 119.9% ($r^2 > 0.980$ for all reactions). Normalized expression of target genes were quantified using the $2^{-\Delta Ct}$ method based on mean cycle thresholds (Ct) (Schmittgen and Livak, 2008). Comparison of relative gene expression, body mass, and organ indices between treatment groups was conducted using one-way ANOVA, or Welch's test when variances significantly differed (DeBeuckelaer, 1996). Observed differences from ANOVA results were followed by Dunnett's comparison test relative to control. Statistical significance was assumed at $p < 0.05$.

Sample extraction and LC-MS/MS analysis. Steroids were extracted from 100 mL water samples using online solid phase extraction (SPE) using a Spark Holland Symbiosys Environ automated extraction system with 500 ng/L testosterone-d_3, $^{13}C_6$-estradiol, and 17α-methyltestosterone as a surrogate. Microwave-assisted solvent extraction (MASE) was used for all sediment samples using 25 ng of the same surogates. Briefly, 2−3 g of sample was weighed into a 10 mL Teflon microwave digestion vessel, mixed with 1 mg of butylated hydroxytoluene and 5 mL of high purity methanol prior to microwaving in a CEM MARS Xpress microwave at 1000 W for 10 min.

Table 4.3. List of method compounds by retention times, with multiple reaction monitoring (MRM) transitions, optimized cone voltage, and collision energies. (From Snow et al., 2013).

Analyte	Mass Transition	Cone (V)	Collision (eV)	MASE extracts Retention Time (min)	On-Line SPE Retention Time (min)
Estriol	288 > 146	22	22	7.60	8.71
11-Ketotestosterone	303 > 121	30	22	8.86	10.21
β-Zearalanol	305 > 189	28	20	9.34	10.76
Androstenedienedione	285 > 121	20	25	9.58	11.08
β-Zearalenol	303 > 285	24	12	9.65	11.31
17β-Trenbolone	271 > 199	32	20	9.89	11.63
α-Zearalanol	305 > 189	28	20	10.29	12.42
17α-Trenbolone	271 > 253	32	20	10.29	12.26
17β-Estradiol	255 > 159	24	20	10.52	12.66
17α-Ethynylestradiol	279 > 133	20	18	10.60	12.81
4-Androstenedione	287 > 97	30	20	10.60	12.66
α-Zearalenol	303 > 285	24	12	10.68	12.89
Estrone	271 > 133	24	20	10.68	12.89
17α-Estradiol	255 > 159	24	20	11.00	13.37
Testosterone	289 > 97	32	24	11.21	13.68
17α-Hydroxyprogesterone	331 > 97	30	25	11.55	14.16
Epitestosterone	289 > 109	32	26	12.58	15.50
Melengestrol Acetate	397 > 337	24	14	14.08	17.31
Progesterone	315 > 97	30	20	14.23	17.47
Androsterone	273 > 255	25	14	14.79	18.02
Internal standards and surrogates					
17β-Estradiol-$^{13}C_6$	261 > 159	24	20	10.52	12.66
Testosterone-d5	294 > 100	32	24	11.20	13.60
α-Methyltestosterone	303 > 97	32	24	12.10	14.87

All extracts were analyzed for steroids using liquid chromatography tandem mass spectrometry with atmospheric pressure photoionization (APPI) utilizing multiple reaction monitoring (MRM) with argon collision gas as detailed in Snow et al. (2013). Briefly, a Thermo HyPurity C18 column (250 × 2 mm, 5 um, 50 °C) was used for gradient separation at a flow rate of 0.35 mL/min. The gradient consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol), with 0 to 3 min at 50% B, 3 to 14 min at 65% B, and 14−20 min at 95% B, with a return to initial

solvent conditions for the last 10 min of the gradient (30 min total). Instrument control, data acquisition and evaluation used MassLynx 4.0 software (Waters Corporation, Milford, MA). Identification of target compounds was accomplished by comparing the retention times for the respective MRM transition in a sample to that of a standard analyzed under the same conditions (Table 4.3). Retention times were considered to match if they were within ±5% of the standards. Testosterone-d_3 was used for quantification of androgens, melengesterol acetate and progesterone, whereas $^{13}C_6$-estradiol served as the internal standard for steroid estrogens in both the online SPE and MASE LC-tandem MS methods. Recovery of the synthetic androgen (17α-methyltestosterone) surrogate was calculated to evaluate individual sample extraction efficiency and possible matrix effects, but analyte concentrations were not corrected for surrogate recovery. Recovery from laboratory fortified sediment blanks was 74% for Pr, 58% for T, 86% for androstenedienedione, 73% for 4-androstenedione. Recovery of laboratory fortified blanks for aqueous compounds ranged between 80-106% for all compounds detected (Tables 4.4 and 4.5).

Table 4.4. Lab reagent blank, lab fortified blank, and duplicate sediment extract samples analyzed using LC-MS/MS. Recoveries (=100xmeasured/fortified) of Laboratory Fortified Blank (LFB) samples prepared and analyzed.

Sample Type	4-Androstenedione	Androstenedienedione	Androsterone	a-Trenbolone	a-Zearalanol	a-Zearalenol	b-Estradiol	b-Trenbolone	b-Zearalanol	b-Zearalenol	Progesterone	Testosterone
LFB	73.27	86.44	88.78	55.58	104.86	114.11	105.47	62.81	104.72	91.38	73.97	85.29
LRB	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Duplicate	0.14						0.40				57.28	
	0.16										56.97	

Table 4.5. Lab reagent blank, lab fortified blank, and duplicate water samples analyzed using LC-MS/MS. Recoveries (=100xmeasured/fortified) of Laboratory Fortified Blank (LFB) samples prepared and analyzed.

Sample Type	4-Androstenedione	Androstanedienedione	Androsterone	b-Trenbolone	Trendione	a-Zearalanol	b-Estradiol	Progesterone	Testosterone	Testolactone
LFB	80.048	89.668	68.772	96.488	77.384	70.628	57.504	80.480	96.688	106.212
LRB	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Duplicate	0.115							1.109	1.114	
	0.250	0.344						1.759	1.336	

Steroid degradation microcosms. Batch experiments using a combination of non-labeled and ^{14}C- radiolabeled Pr were conducted to determine detailed degradation profiles and metabolite generation within the sediment/water systems. The ^{14}C- radiolabel acted as a tracer to verify microcosms were designed such that the sediment to water ratio (approximately 1g :5.6 mL), lighting regime (16:8 light:dark), and temperature (26 $\pm$ 0.2 ^{o}C) would be the similar to that used in the fish exposure experiments. The equilibration procedure differed slightly between the degradation microcosms and the exposure aquaria in order to gain detailed measurements. From past experience, it was expected that the system would be susceptible to rapid changes for the first 24 – 48 hours. In order to see these changes and since manipulation of the microcosms was not limited by scale, the entire system was rapidly assembled and the data presented from the first 24 hours in the microcosms would correlate to the equilibration period of the fish exposure aquaria. Each steroid was delivered to experimental microcosms using a stock solution of 250 mg/L Pr in ethanol. Solvent was evaporated from each microcosm under a steady stream of nitrogen before the addition of water and sediment to eliminate the possibility of solvent

effects. Each microcosm consisted of 3 g of sediment spiked with 500 ng/g dw Pr in 17 mL of distilled deionized water in 50 mL glass centrifuge tubes. Microcosms were opened for 30 min each day to assure aerobic conditions. This was verified using two additional reactors containing resazurin dye. Microcosms were sacrificed at 0 hr, 6 hr, 12 hr, 18 hr, 24 hr, 36 hr, and daily from day 2 to day 14. Sediment and aqueous phases of each microcosm were separated by centrifugation at 2000 rpm for 15 min. Steroids were extracted from aqueous samples upon collection using the previously described methods with resulting extracts along with sediment phase samples stored at -20°C until further analysis. The same experimental design was used for both sediments (silty loam and sand) with both aqueous and sediment phase of microcosm assessed for concentrations of parent steroid and metabolites.

Characterization of steroid hormone transformation products using LC/MS and radioactivity detection. Extracted steroids from the microcosm experiments were separated by a water/methanol gradient (0.4 ml/min) on a 2695 HPLC (Waters, Milford, MA) equipped with a HyPurity C18 column (Thermo Scientific, Waltham, MA) at 50°C and using an injection volume of 50 µL.

Both UV (230 nm, 486 MS, Waters, Milford, MA) and β-particle (β-RAM, IN/US Systems, Tampa, FL) ^{14}C radioactivity detectors were used to produce chromatograms for each extract injection and peaks compared to retention times measured using an ion trap mass spectrometer. The mobile phase gradient was optimized for steroid hormone separation. A Thermo LCQ ion trap mass spectrometer (San Jose, CA) was used to characterize degradation products in positive full scan mode from 150 to 450 amu with

atmospheric pressure chemical ionization (APCI) source. Source conditions were: Vaporizer temperature: 450°C; Sheath gas: 80 (arbitrary units); Auxiliary gas: 1 (arbitrary units); Discharge current: 9.00 µA; Capillary temperature: 200°C; Capillary voltage: 6.00 V; and Tube Lens offset: -15.00 V. The β-Ram radioactivity detector was used to identify regions of each chromatogram likely to contain transformation products. Peaks in the radioactivity chromatogram for each sample were compared to the MS spectrum taken at approximately the same time to determine the identity of the radio-labeled compound eluting during that time. Steroid concentrations were quantified by internal standards from full scan (150-440 amu) APCI MS data (LCQ, Thermo Finnigan, San Jose, CA) with the Xcalibur software (version 1.3, Thermo Finnigan, San Jose, CA) using a five-point linear regression (50-2000 µg/L).

4.3 RESULTS AND DISCUSSION

The goal of this study was to conduct an integrated evaluation of the fate of Pr in sediment and water to understand the corresponding bioavailability. This was accomplished by using two natural aquatic sediments to determine if fathead minnows exposed to sediment contaminated with Pr would exhibit altered endocrine function, while measuring degradation and metabolite formation of Pr over the course of the experiment in both the exposure aquaria and in controlled microcosms. Results from this study indicate that Pr degradation will result in known androgens and an anti-estrogen and confirms Pr exposure will result in defeminization of female fathead minnows. While the current study was not designed to evaluate the mechanism of steroid exposure, the effects measured in the fish coupled with analytical chemistry data from both the

exposure aquaria and microcosms suggests that fish exposed to Pr associated with sand exhibit a biological response in response to aqueous steroids; while fish exposed to Pr associated with silty loam sediment exhibit biological effects consistent with exposure to the dominant sediment associated compound.

Progesterone degradation in controlled microcosms results in androgens and an anti-estrogen. In the microcosms (Figure 4.1), steroids were predominantly detected in the sediment phase with sediment-associated Pr as the dominant compound in both the sand and silty loam. In the sand microcosms, there was rapid transformation of Pr to T within the first 6 hr in both the aqueous and sediment phases with sediment-associated androsteinedione, T, and androstadienedione appearing around 10 d. In the silty loam microcosms, there were measurable albeit low levels of androgen throughout the 14 d period with an increase in androstadienedione measured at 14 d (Figure 4.1B). The silty loam microcosms also had a greater conservation of mass after 14 d (24%) compared to the sand microcosms (18%) after 14 d (Figure 4.1). Additionally, testolactone, an anti-estrogen, was frequently detected in both the water and sediment phases within the microcosms at concentrations ranging from 0.05 – 0.11µg/L and 0.6 – 2.3 ng/g in the sand and 0.10 – 0.15 µg/L and 0.59 – 2.3 ng/g in the silty loam microcosms, respectively. The degradation microcosms in this study used ^{14}C radio-labeled progesterone as a tracer. Extracts from both water and sediments were processed using LC/MS and radioactivity detection in tandem. Both instruments were run simultaneously using the same protocol. Peaks in radioactivity were compared to LC/MS readings from the same time to determine the identity of the compound. We did not detect peaks in radioactivity that would correspond to any other unknown compounds. While previous studies have

evaluated Pr degradation using pure cultures in enriched matrices (Bartmanska et al., 2005; Hunter and Carragher, 2003; Hunter et al., 2009; Mostafa and Zohri, 2000; Yildirim et al., 2011), this confirms that Pr degradation will result in the production of several known androgens and at least one antiestrogen in more complex systems.

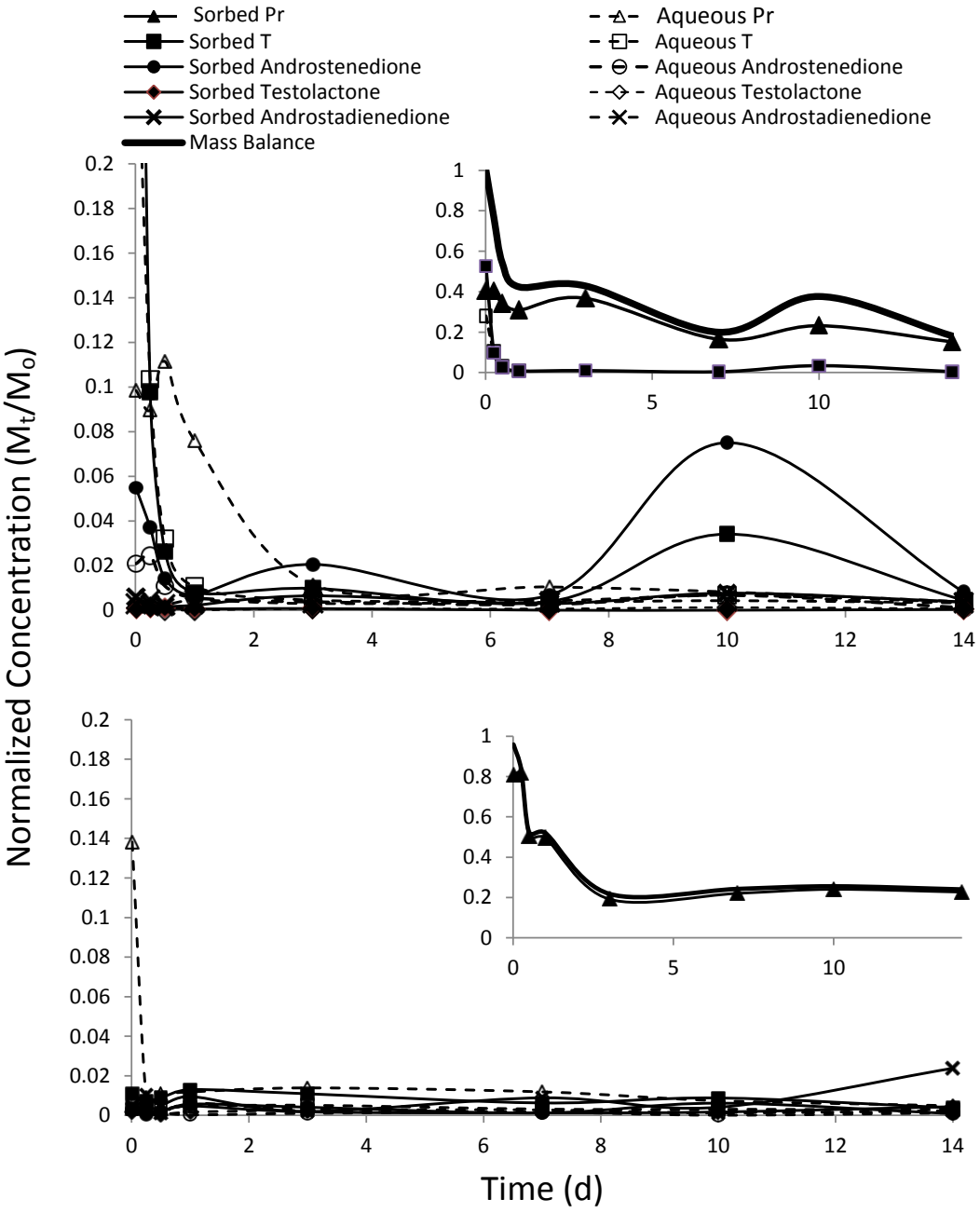


Figure 4.1. Normalized concentrations expressed as Mt/Mo from microcosms spiked at 500 ng/g progesterone containing A) sand and B) silty loam sediments. For each sediment, normalized concentrations of steroids $\leq$ 0.2 are presented with mass balance and steroids with normalized concentrations exceeding 0.2 as insets. Solid lines represent sediment-associated steroids. Dashed lines indicate steroids measured in the aqueous phase.

Effects measured in female fatheads minnows exposed to progesterone associated with sandy sediment are likely due to desorption and transformation. Fish exposed to Pr associated with the sandy sediment exhibited significant decreases ($p=0.0159$) in the expression of hepatic Vtg in the 5 and 50 ng/g treatments groups when compared to the control sediment after 7 d of exposure (Figure 4.2A) with no significant differences in the expression of AR (Figure 4.2C) or ER-α . By 14 d, fish exposed to Pr associated with the sandy sediment exhibited significant decreases in the expression of Vtg ($p=0.003$) in the 50 and 500 ng/g treatment group (Figure 4.2B) with a corresponding decrease in the expression of AR ($p=0.006$) (Figure 4.2D). It is common for exposure to endocrine disruptors including steroids to cause a non-monotonic dose response in organisms. When evaluating fathead minnows exposed to aqueous progesterone for 21 days, a similar trend was observed where the female fish exposed to 10 and 100 ng/L progesterone had significant reductions in the expression of Vtg; while the Vtg measured in fish exposed to 1000 ng/L was not significantly different from the negative control. There were no significant differences found in morphometric data for either male or female fish regardless of the exposure regime (Table 4.2). The reduction in Vtg expression is consistent with Pr exposure as previous studies using female fathead minnows exposed to aqueous Pr documented reduced expression of Vtg (DeQuattro et al., 2012); however, exposure to androgens would lower Vtg expression in female fish as well. While prior studies evaluating aqueous exposures of fish to progesterone did not assess AR response (DeQuattro et al., 2012), *ex vivo* studies evaluating fat head minnow ovaries (Chishti et al., 2014) and *in vitro* studies evaluating nuclear AR (Ellestad et al., 2014) have shown Pr acts as a weak AR agonist.

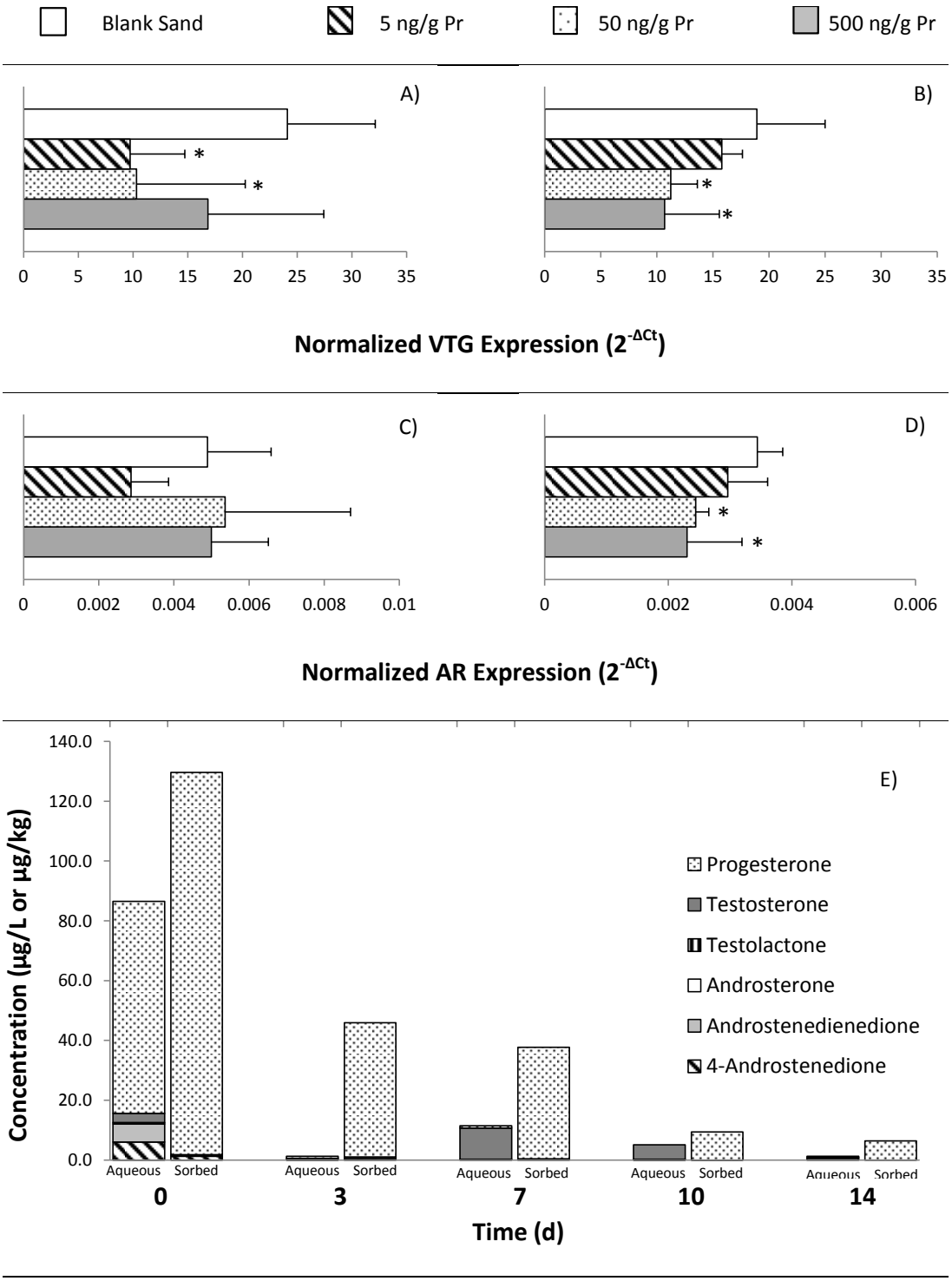


Figure 4.2. Normalized hepatic expression (X ± sd) of vitellogenin (VTG) and androgen receptor (AR) of female fathead minnows exposed to sand-associated progesterone after 7 (panels A and C) and 14 (panels B and D) day exposures with measured sorbed and aqueous phase concentrations (panel E) obtained during periodic grab samples . * Denotes significant differences compared to blank sediment group.

Measured concentrations of sorbed and aqueous phase steroids within the exposure aquaria spiked at 500 ng/g Pr are shown in Figure 2E with additional details in Table 4.6. Through the duration of the 14-d exposure, sediment-associated Pr persisted and, with exception of day 10, was also measured in the aqueous phase within the aquaria. Interestingly, androsterone was not detected in the microcosms for either sediment (Figure 4.1), but was found in the aqueous phase at day 10 in the sand exposure aquaria (Figure 2E). Steroids were detected in the aqueous phase for longer time periods compared with our previous laboratory studies assessing sediment-associated steroids (Sangster et al., 2014; Sellin et al., 2010), however, this may be due in part to the higher initial Pr concentration evaluated in the present study. It is also important to note that co-extracted matrix in sediment extracts from the exposure aquaria produced high levels of background noise that may have limited the detection of several compounds (Table 4.6). Steroids were detected in aquaria containing the 5 ng/g and 50 ng/g-amended sediments (data not shown); however rapid degradation was observed with steroid concentrations approaching the detection limits by 3 days with minimal detection of metabolites over the exposure period.

Table 4.6. Measured sorbed and aqueous phase steroid concentrations in 500 ng/g progesterone exposure aquaria. Days refer to time of fish exposure and do not reflect initial equillibration period prior to the addition of fish. Shaded cells represent the movement of steroid based on equillibrium calcualtions (i.e. shaded sediment phase represents sorption and shaded water phase represents desorption).

		Day 0		Day 3		Day 7		Day 10		Day 14	
	Compound	Water (µg/L)	Sediment (µg/kg)	Water (µg/L)	Sediment (µg/kg)	Water (µg/L)	Sediment (µg/kg)	Water (µg/L)	Sediment (µg/kg)	Water (µg/L)	Sediment (µg/kg)
Sand	Progesterone	70.93	127.85	0.68	44.84	0.78	37.30		9.32	0.33	6.42
	4-Androstenedione	6.05	1.28	0.11	0.50	0.30	0.31	0.03		0.23	
	Androstenedienedione	6.07	0.41		0.59		0.13		0.12	0.53	
	Androsterone							0.08			
	Testosterone	3.08	0.11	0.49		10.39		4.82		0.22	
	Testolactone	0.44						0.16			
	Mass Balance (M_t/M_o)	1.25		0.11		0.22		0.08		0.03	
Silty Loam	Progesterone	48.029	281.15	0.171	191.64	1.167	111.66	0.014	34.48	0.632	22.33
	4-Androstenedione	0.361	0.22	0.128	0.20	0.204	0.32	0.013		0.048	
	Androstenedienedione	0.731						0.269			
	Testosterone	0.372		0.128		0.646				0.041	
	Testolactone					0.288					
	Mass Balance (M_t/M_o)	1.12		0.40		0.26		0.09		0.05	

Based on equilibrium partitioning calculations using log K_{oc} values either determined experimentally using the sediments in this study (Sangster et al., 2015) or reported previously (Table 4.1), desorption of Pr from the sand is expected between days 3 and 10 (Table 4.6). Interestingly, there is an initial peak of aqueous Pr and the androgens, T, androstenedienedione, and 4-androstenedione, that disappears rapidly (Figure 4.2E) with another increase in aqueous concentrations of T and 4-androstenedione beginning at 7 days coinciding with the predicted movement of Pr from the sediment to the aqueous phase. This suggests that the association between the sediment and parent compound, Pr, acts to preserve the compound with transformation occurring in the aqueous phase. Additionally, we would expect movement of steroids, Pr, 4-androstenedione, androstenedienedione, T, and testolactone from the aqueous phase to the sediment at day 0 and 14 (Table 4.6). However, the aquaria data show that with the exception of androsteinedienedione, sediment concentrations continue to decrease relative to the aqueous phase.

Taken together, it is likely that the effects measured in female fish exposed to Pr associated with the sandy sediment are predominantly in response to aqueous steroid in the system. The initial concentration of aqueous Pr coupled with the presence of androgenic and anti-estrogenic compounds is such that Vtg is significantly reduced; however, the androgens are not present either at high enough concentrations or for a long enough period to cause significant differences in AR response. After 14 days of exposure, the female fish respond to the increase in aqueous T evidenced by both significant difference in Vtg and AR (Figure 4.2B and 4.2D).

Effects measured in female fatheads minnows exposed to progesterone associated with silty loam sediment are likely due to contact with sediment-associated steroid. Female fish exposed to Pr associated with the silty loam sediment showed alteration in gene expression after 14 d of exposure in the 50 and 500 ng/g Pr treatment groups. These fish exhibited significant reductions ($p=0.0039$) in the expression of hepatic Vtg (Figure 4.3B), but not AR (Figure 4.3C and 4.3D) or ER-α. This is in contrast to the fish exposed to Pr in sand where significant reductions in the expression of Vtg were found at both 7 and 14 day, and were also accompanied by reductions in AR expression at 14 days (Figure 4.2). While the reduction in Vtg is consistent with exposure to Pr (DeQuattro et al., 2012), the differential response measured between the two sediment types suggests differences may exist between mechanism of exposure and Pr fate influenced by differences in sediment characteristics.

Measured concentrations of sorbed and aqueous phase steroids within the silty loam exposure aquaria spiked at 500 ng/g P4 are shown in Figure 4.3E with additional details in Table 4.6. Pr was detected in both the aqueous and sediment-associated phase consistently during the 14-day exposure. The only other sediment-associated steroid detected in the exposure aquaria was 4-androstenedione during days 0, 3, and 7. The androgens, 4-androstenedione, androstenedienedione, and T, along with the

anti-estrogen, testolactone, were detected in the aqueous phase of the exposure aquaria at low (< 0.731 µg/L) but measurable concentrations (Table 4.6). Androsterone was not detected in the silty loam exposure aquaria at any time point evaluated (Table 4.6).

Sediment-associated Pr persisted over the 14 d period (Figure 4.3E) with conservation of mass (above 26% in the first 7 d) well above that from the sand aquaria (Table 4.6). This is not surprising as Pr sorbs more readily to the silty loam sediment (K_d = 207.2 L/kg) as compared to the sand (K_d = 33.3 L/kg) (Sangster et al., 2015). Desorption of Pr from the silty loam is expected based on equilibrium partitioning calculations at days 3 and 10 (Table 4.6) and was followed by an increase in the aqueous concentration of T and 4-androstenedione on days 7 and 14. Interestingly, aqueous concentrations of T peak at 7 d in both the sand and silty loam sediments at 10.39 µg/L and 0.646 µg/L, respectively. In both instances, this was proceeded by expected of desorption of P4 from the sediments at day 3 (Table 4.6).

For both sediments, over 60% of sediment-associated Pr may be found in the finer particles (approximately 24% by weight in the silty loam and 8% by weight in the sand) within the whole sediment (Sangster et al., 2015). As finer particles are more likely to be suspended in the water column, contact with fish would be greater in the silty loam sediments. Even with higher concentrations of sediment-associated Pr measured in the silty loam exposure aquaria (Figure 4.3E), biological effects were not seen until 14 d of exposure (Figure 4.3B). The lack of significant AR response coupled with the limited detection of aqueous steroids suggest the significant reduction of Vtg after 14 days of exposure (Figure 4.3B) is due to low dose exposure either from sediment-associated steroid or small amounts of aqueous steroid desorbed from the sediments over the 14 day period.

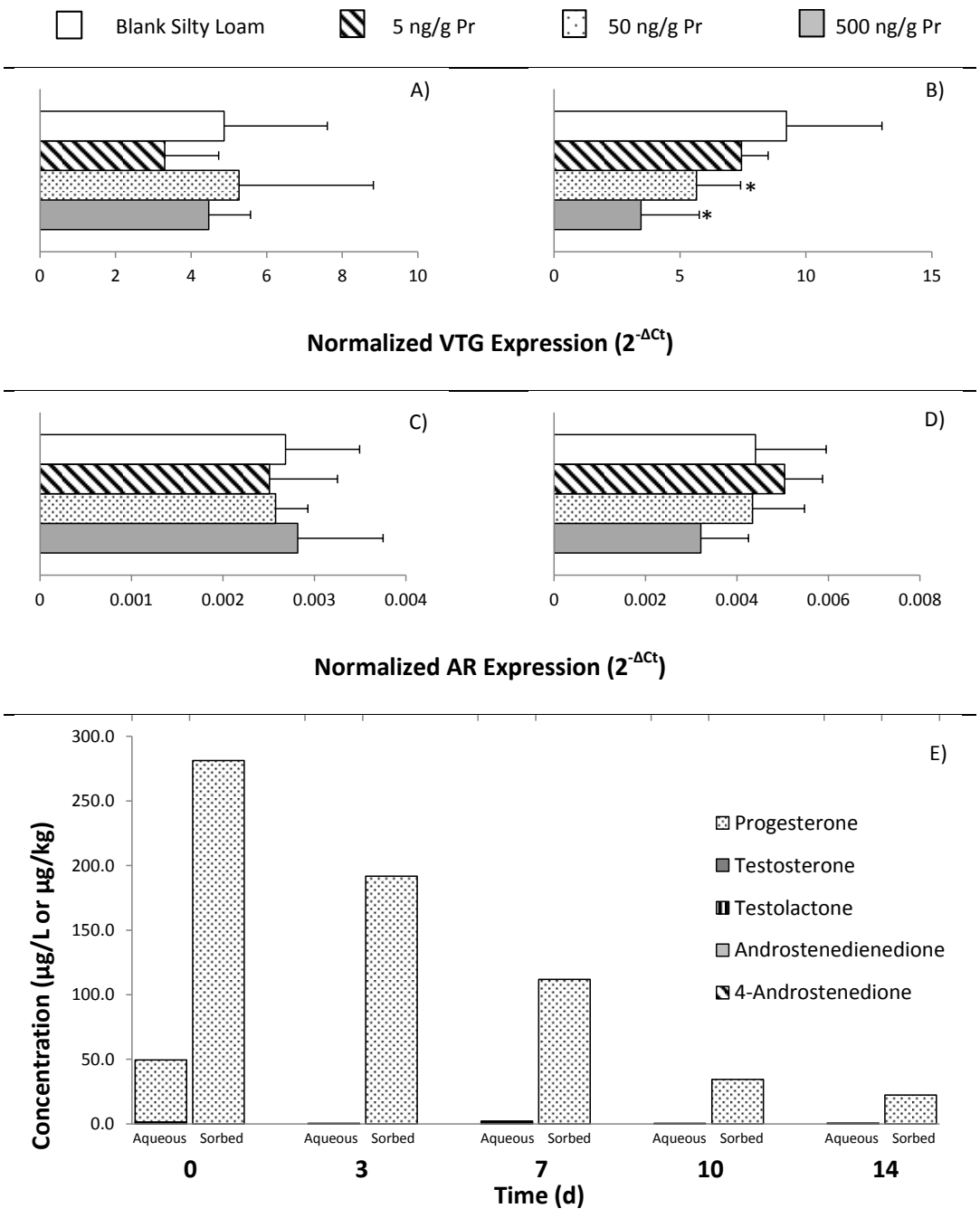


Figure 4.3. Normalized hepatic expression (X ± sd) of vitellogenin (VTG) and androgen receptor (AR) of female fathead minnows exposed to silty loam-associated progesterone after 7 (panels A and C) and 14 (panels B and D) day exposures with measured sorbed and aqueous phase concentrations (panel E) obtained during periodic grab samples . * Denotes significant differences compared to blank sediment group.

Environmental implications of sediment-associated progesterone in aquatic systems. In systems subject to episodic loading of steroid hormones, the importance of sediment/contaminant interactions on both contaminant fate and the subsequent bioavailability to aquatic organisms needs to be considered when determining environmental risk associated with a contaminant. The results of this study, as well as those reported in Sangster et al.(2014), illustrates the effect sediment type has on compound fate and bioavailability in aquatic systems. In both sediments evaluated in this study, Pr degradation resulted in the androgens, androsteinedione, T, and androstadienedione, and the anti-estrogen testolactone confirming results of previous work using pure cultures is applicable in more complex environmentally relevant systems. The finer texture silty loam sediment allows for greater persistence of the compounds and may provide for increased transport and contact by providing greater quantities of suspended particles; however, biological response appeared to be mitigated by steroid interactions with the silty loam sediment. The coarser sand sediment composition allowed for persistence of Pr while providing an immediate source for biologically active compounds to the aqueous phase. This suggests the occurrence of steroid hormones in sediment rather than water alone should be assessed to determine the environmental risks in impacted systems.

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CHAPTER 5: FUTURE DIRECTIONS RELATED TO THE OCCURRENCE, FATE, AND BIOLOGICAL EFFECTS OF STEROIDS IN AQUATIC ENVIRONMENTS

To date, there has been a significant amount of research detailing the occurrence of steroids in aquatic systems and biological effects stemming from aqueous concentrations of various compounds. The fate of steroids has been evaluated as it relates to the degradation and transport through agricultural soils, wastewater treatment applications, and to a lesser extent, in aquatic systems. However, there is limited information linking the occurrence, fate, and biological effects of steroids in sediment-water systems to truly identify the risk these compounds pose. Therefore, further research efforts in the field will need to provide a holistic approach using each aspect to gain information to understand how occurrence, fate, and biological response are related. The following details gaps in knowledge and questions that stem directly from the research presented in this document, as well as, future research opportunities in the field.

One of the more challenging knowledge gaps faced in steroid fate and exposure experiments has been the ability to determine environmentally relevant sediment concentrations in aquatic systems. The vast majority of papers detailing the occurrence of steroids in the environment only evaluate aqueous concentrations. There are a limited number of published papers evaluating steroid occurrence in sediments. This makes determining environmentally relevant levels of contamination challenging at best. In turn, it becomes difficult to determine possible biological effects stemming from exposure to the parent compound or to the suite of compounds that may be present as the parent compound degrades. In light of the growing body of work implicating steroid-sediment

interactions as a source of biologically active compounds to the aquatic environment, it becomes increasingly important to have a baseline of environmental contamination of these compounds.

As we have seen in previous projects evaluating the fate and biological effects stemming from sediment associated steroids, the parent compound will degrade at a fairly consistent rate to consistent metabolites regardless of the scale of the experiment. The increase in scale from microcosms to exposure experiments seems to affect the phase (sediment or water) the compounds are detected in more so than degradation rates or metabolites formed. We see differential response in the fish when considering different sediments. However, it is uncertain if these differences in effects are in response to differences in compound fate and/or mechanisms of exposure. This may prove to be different for each steroid and sediment combination as each steroid may exhibit slightly different sorption affinities and degradation rates depending on the concentration evaluated and the type of sediment used. Additionally, there is research showing fish may have an enhanced biological response to intermittent or pulsed exposure to aqueous steroids above what is expected from exposure to a constant aqueous concentration. It is possible that the biological response we have documented may simply be in response to "pulses" as the parent compound transforms into various biologically active compounds.

One idea that this dissertation has touched upon is the biological response of organisms to complex mixtures. As we design exposure experiments, we begin with a parent compound. The parent compound degrades to form metabolites with different efficacies. While work to date explicitly evaluating biological response to complex mixtures is limited, a common challenge appears to be the choice of endpoints. It is

becoming more apparent that relevant endpoints need to be expanded to include both varying levels of organization and during different life stages to be able to begin to understand the effects caused by these mixtures. However, this may prove to be more important when working with solutions containing contaminants with differing modes of action within the target organism.

Agriculturally impacted waterways have the potential for a diverse collection of contaminants including steroids, herbicides, pesticides, and antibiotics. In areas impacted by animal agricultural wastes, it would be expected that both steroids and antibiotics would be found concurrently. There is evidence that antibiotics have the potential to affect soil microbes and this, in turn, can slow the microbial degradation of steroids in agricultural soils. Evaluating the effects of antibiotics on steroid fate in aquatic systems may prove interesting and have important impacts in aquatic systems and beyond.

REFERENCES

Ankley GT, Jensen KM, Makynen EA, Kahl MD, Korte JJ, Hornung MW et al. Effects of the Androgenic Growth Promoter 17-β-Trenbolone on Fecundity and Reproductive Endocrinology of the Fathead Minnow. Environmental Toxicology & Chemistry 2003;22:1350-60.

Arnon S, Dahan O, Elhanany S, Cohen K, Pankratov I, Gross A et al. Transport of testosterone and estrogen from dairy-farm waste lagoons to groundwater. Environ Sci Technol 2008;42:5521-6.

Atteke C, Vetillard A, Fostier A, Garnier DH, Jego P, Bailhache T. Effects of progesterone and estradiol on the reproductive axis in immature diploid and triploid rainbow trout. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology 2003;134:693-705.

Bartelt-Hunt S, Snow DD, Damon-Powell T, Miesbach D. Occurrence of steroid hormones and antibiotics in shallow groundwater impacted by livestock waste control facilities. J Contam Hydrol 2011;123:94-103.

Bartelt-Hunt S, Snow DD, Kranz WL, Mader TL, Shapiro CA, van Donk SJ et al. Effect of Growth Promotants on the Occurrence of Endogenous and Synthetic Steroid Hormones on Feedlot Soils and in Runoff from Beef Cattle Feeding Operations. Environ Sci Technol 2012;46:1352-60.

Bartmanska A, Dmochowska-Gladysz J, Huszcza E. Steroids' transformations in Penicillium notatum culture. Steroids 2005;70:193-8.

Bonin JL, Simpson MJ. Sorption of steroid estrogens to soil and soil constituents in single- and multi-sorbate systems. Environmental Toxicology and Chemistry 2007;26:2604-10.

Braga O, Smythe G, Schaefer A, Feitz A. Steroid estrogens in ocean sediments. Chemosphere 2005;61(6):827-833.

Card ML, Chin Y, Lee LS, Khan B. Prediction and Experimental Evaluation of Soil Sorption by Natural Hormones and Hormone Mimics. J Agric Food Chem 2012;60:1480-7.

Casey FXM, Larsen GL, Hakk H, Simunek J. Fate and transport of 17 beta-estradiol in soil-water systems. Environ Sci Technol 2003;37:2400-9.

Casey FXM, Simunek J, Lee J, Larsen GL, Hakk H. Sorption, mobility, and transformation of estrogenic hormones in natural soil. J Environ Qual 2005;34:1372-9.

Casey FXM, Hakk H, Sim&uaring, nek J, Larsent GL. Fate and Transport of Testosterone in Agricultural Soils. Environ Sci Technol 2004;38:790-8.

Casey FXM, Ocluor PG, Hakk H, Larsen GL, DeSutter TM. Transport of 17 beta-estradiol and testosterone in a field lysimeter. Soil Sci 2008;173:456-67.

Chishti YZ, Feswick A, Martyniuk CJ. Progesterone increases ex vivo testosterone production and decreases the expression of progestin receptors and steroidogenic enzymes in the fathead minnow (Pimephales promelas) ovary. Gen Comp Endocrinol 2014;199:16-25.

Chishti YZ, Feswick A, Munkittrick KR, Martyniuk CJ. Transcriptomic profiling of progesterone in the male fathead minnow (Pimephales promelas) testis. Gen Comp Endocrinol 2013;192:115-25.

Chun S, Lee J, Radosevich M, White DC, Geyer R. Influence of agricultural antibiotics and 17 beta-estradiol on the microbial community of soil. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 2006;41:923-35.

Dane JH, Topp GC. Methods of Soil Analysis: Part 4 - Physical Methods 2002;5:1692.

Das BS, Lee LS, Rao PSC, Hultgren RP. Sorption and degradation of steroid hormones in soils during transport: Column studies and model evaluation. Environ Sci Technol 2004;38:1460-70.

DeBeuckelaer A. A closer examination on some parametric alternatives to the ANOVA F-test. Statistical Papers 1996;37:291-305.

DeQuattro ZA, Peissig EJ, Antkiewicz DS, Lundgren EJ, Hedman CJ, Hemming JDC et al. Effects of progesterone on reproduction and embryonic development in the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 2012;31:851-6.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening. Environmental science & technology 1998;32(11):1549-1558.

Duong CN, Ra JS, Schlenk D, Kim SD, Choi HK, Kim SD. Sorption of Estrogens onto Different Fractions of Sediment and Its Effect on Vitellogenin Expression in Male Japanese Medaka. Arch Environ Contam Toxicol 2010;59:147-156.

Duong CN, Ra JS, Cho J, Kim SD, Choi HK, Park J et al. Estrogenic chemicals and estrogenicity in river waters of South Korea and seven Asian countries. Chemosphere 2010a;78:286-93.

Duong CN, Schlenk D, Chang NI, Kim SD. The effect of particle size on the bioavailability of estrogenic chemicals from sediments. Chemosphere 2009;76:395-401.

Durhan EJ, Lambright CS, Makynen EA, Lazorchak JM, Hartig PC, Wilson VS et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environmental health perspectives 2006;114:65-68.

Ellestad LE, Cardon M, Chambers IG, Farmer JL, Hartig P, Stevens K et al. Environmental Gestagens Activate Fathead Minnow (Pimephales promelas) Nuclear Progesterone and Androgen Receptors in Vitro. Environ Sci Technol 2014;48:8179-87.

Fan Z, Wu S, Chang H, Hu J. Behaviors of Glucocorticoids, Androgens and Progestogens in a Municipal Sewage Treatment Plant: Comparison to Estrogens. Environ Sci Technol 2011;45:2725-33.

Gall HE, Sassman SA, Lee LS, Jafvert CT. Hormone discharges from a midwest tile-drained agrecosystem receiving animal wastes. Environ Sci Technol 2011;45:8755-64.

Gineys N, Giroud B, Gineys M, Vulliet E. Retention of selected steroids on a silt-loam soil. Journal of Environmental Science and Health Part A-Toxic/hazardous Substances & Environmental Engineering 2012;47:2133-40.

He C, Bartholdy J, Christiansen C. Clay mineralogy, grain size distribution and their correlations with trace metals in the salt marsh sediments of the Skallingen barrier spit, Danish Wadden Sea. Environmental Earth Sciences 2012;67:759-69.

Hunter AC, Carragher NE. Flexibility of the endogenous progesterone lactonisation pathway in Aspergillus tamarii KITA: transformation of a series of cortical steroid analogues. J Steroid Biochem Mol Biol 2003;87:301-8.

Hunter AC, Coyle E, Morse F, Dedi C, Dodd HT, Koussoroplis S. Transformation of 5-ene steroids by the fungus Aspergillus tamarii KITA: Mixed molecular fate in lactonization and hydroxylation pathways with identification of a putative 3 beta-hydroxy-steroid dehydrogenase/Delta(5)-Delta(4) isomerase pathway. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 2009;1791:110-7.

Islam K, Weil R. A rapid microwave digestion method for colorimetric measurement of soil organic carbon. Commun Soil Sci Plant Anal 1998;29:2269-84.

Jeffries MKS, Conoan NH, Cox MB, Sangster JL, Balsiger HA, Bridges AA et al. The anti-estrogenic activity of sediments from agriculturally intense watersheds: Assessment using in vivo and in vitro assays. Aquatic Toxicology 2011;105:189-98.

Jenkins RL, Wilson EM, Angus RA, Howell WM, Kirk M. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicological Sciences 2003;73:53-9.

Jessick AM, Skolness S, Kolok AS. Sandy sediment and the bioavailability of 17B-trenbolone to adult female fathead minnows
. Aquat Toxicol 2014;148:48-54.

Jobling S, Sheahan D, Osborne JA, Mattheissen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals. Environmental Toxicology & Chemistry 1996;15(2):194-202.

Johnson A, White C, Besien T, Jurgens M. The sorption potential of octylphenol, a xenobiotic oestrogen, to suspended and bed sediments collected from industrial and rural reaches of three English rivers. Science of the Total Environment 1998;210-211:271-82.

Jurgens M, Holthaus K, Johnson A, Smith J. The potential for estradiol and ethynylestradiol degradation in English rivers. Environmental Toxicology & Chemistry 2002;21:480-488.

Khan B, Lee LS. Soil temperature and moisture effects on the persistence of synthetic androgen 17α-trenbolone, 17β-trenbolone and trendione. Chemosphere 2010;79:873-9.

Khan B, Lee LS, Sassman SA. Degradation of Synthetic Androgens 17α- and 17β-Trenholone and Trendione in Agricultural Soils. Environ Sci Technol 2008;42:3570-4.

Khan B, Qiao X, Lee LS. Stereoselective Sorption by Agricultural Soils and Liquid--Liquid Partitioning of Trenbolone (17α and 17β) and Trendione. Environ Sci Technol 2009;43:8827-33.

Kim I, Yu Z, Xiao B, Huang W. Sorption of Male Hormones by Soils and Sediments. Environmental Toxicology & Chemistry 2007;26:264-70.

Kolodziej EP, Gray JL, Sedlak DL. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environmental Toxicology & Chemistry 2003;22:2622-2629.

Kolodziej EP, Sedlak DL. Rangeland grazing as a source of steroid hormones to surface waters. Environ Sci Technol 2007;41:3514-20.

Kolok AS, Sellin MK. The environmental impact of growth promoters employed by the United States beef cattle industry: History, current knowledge, and future directions. Reviews of Environmental Contamination and Toxicology 2008;195:1-30.

Kolok AS, Snow DD, Kohno S, Sellin MK, Guillette LJ. Occurrence and biological effect of exogenous steroids in the Elkhorn River, Nebraska, USA. Sci Total Environ 2007;388:104-15.

Kumar V, Johnson AC, Trubiroha A, Tumova J, Ihara M, Grabic R et al. The Challenge Presented by Progestins in Ecotoxicological Research: A Critical Review. Environ Sci Technol 2015;49:2625-38.

Labadie P, Cundy AB, Stone K, Andrews M, Valbonesi S, Hill EM. Evidence for the Migration of Steroidal Estrogens through River Bed Sediments. Environ Sci Technol 2007;41:4299-304.

Lange IG, Daxenberger A, Schiffer B, Witters H, Ibarreta D, Meyer HHD. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal Chim Acta 2002;473:27.

Lee LS, Strock TJ, Sarmah AK, Rao P, Suresh C. Sorption and Dissipation of Testosterone, Estrogens, and Their Primary Transformation Products in Soils and Sediment. Environ Sci Technol 2003;37:2400-2409.

Liu S, Ying G, Zhao J, Chen F, Yang B, Zhou L et al. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography-electrospray ionization tandem mass spectrometry. Journal of Chromatography a 2011;1218:1367-78.

Loffredo E, Senesi N. Fate of anthropogenic organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure and Applied Chemistry 2006;78:947-61.

Lu Z, Zeng F, Xue N, Li F. Occurrence and distribution of polycyclic aromatic hydrocarbons in organo-mineral particles of alluvial sandy soil profiles at a petroleum-contaminated site. Sci Total Environ 2012;433:50-7.

Mashtare ML, Green DA, Lee LS. Biotransformation of 17 alpha- and 17 beta-estradiol in aerobic soils. Chemosphere 2013;90:647-52.

Mostafa ME, Zohri AA. Progesterone side-chain degradation by some species of Aspergillus flavus group. Folia Microbiol (Praha) 2000;45:243-7.

Neale PA, Escher BI, Schafer AI, Neale PA. pH dependence of steroid hormone--organic matter interactions at environmental concentrations. Sci Total Environ 2009;407:1164-73.

Peck M, Gibson R, Koertenkamp A, Hill E. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environmental Toxicology & Chemistry 2004;23:945-952.

Pierard C, Budzinski H, Garrigues P. Grain-size distribution of polychlorobiphenyls in coastal sediments. Environ Sci Technol 1996;30:2776-83.

Pollio A, Pinto G, DellaGreca M, Fiorentino A, Previtera L. Biotransformations of progesterone by Chlorella spp. Phytochemistry 1996;42:685-8.

Qi Y, Zhang TC, Ren Y. Testosterone sorption and desorption: Effects of soil particle size. J Hazard Mater 2014;279:493-501.

Robinson B, Hellou J. Biodegradation of endocrine disrupting compounds in harbor seawater and sediment. Science of the Total Environment 2009;doi:10.1016/j.scitotenv.2009.07.003.

Sangster JL, Oke H, Zhang Y, Bartelt-Hunt SL. The effect of particle size on sorption of estrogens, androgens and progestagens in aquatic sediment. J Hazard Mater 2015;299:112-121.

Sangster JL, Zhang Y, Hernandez R, Garcia YA, Sivils JC, Cox MB et al. Bioavailability and fate of sediment-associated trenbolone and estradiol in aquatic systems. Sci Total Environ 2014;496:576-84.

Sarmah AK, Northcott GL, Scherr FF. Retention of estrogenic steroid hormones by selected New Zealand soils. Environ Int 2008;34:749-55.

Schiffer B, Daxenberger A, Meyer K, Meyer HHD. The Fate of Trenbolone Acetate and Melengestrol Acetate after Application as Growth Promoters in Cattle: Environmental Studies. Environ Health Perspect 2001;109:1145.

Schiffer B, Totsche KU, Jann S, Kögel-Knabner I, Meyer K, Meyer HHD. Mobility of the growth promoters trenbolone and melengestrol acetate in agricultural soil: column studies. Sci Total Environ 2004;326:225-37.

Scherr FF, Sarmah AK, Di HJ, Cameron KC, Scherr FF. Sorption Of Estrone And Estrone-3-Sulfate From $CaCl^2$ Solution And Artificial Urine In Pastoral Soils Of New Zealand. Environmental Toxicology & Chemistry 2009;28:2564-71.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C-T method. Nature Protocols 2008;3:1101-8.

Scott Mansell D, Bryson RJ, Harter T, Webster JP, Kolodziej EP, Sedlak DL. Fate of Endogenous Steroid Hormones in Steer Feedlots Under Simulated Rainfall-Induced Runoff. Environ Sci Technol 2011;45:8811-8.

Seki M, Fujishima S, Nozaka T, Maeda M, Kobayashi K. Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. Environmental Toxicology & Chemistry 2006;25:2742-52.

Sellin Jeffries MK, Conoan NH, Cox MB, Sangster JL, Balsiger HA, Bridges AA et al. The anti-estrogenic activity of sediments from agriculturally intense watersheds: Assessment using in vivo and in vitro assays. Aquatic Toxicology 2011;105:189-98.

Sellin MK, Snow DD, Kolok AS. Reductions in hepatic vitellogenin and estrogen receptor alpha expression by sediments from an agriculturally impacted waterway. Aquatic Toxicology 2010;96:103-8.

Sellin MK, Snow DD, Schwarz M, Carter BJ, Kolok AS. Agrichemicals in Nebraska, Usa, Watersheds: Occurrence and Endocrine Effects. Environmental Toxicology & Chemistry 2009;28:2443-8.

Seralini GE, Moslemi S. Aromatase inhibitors: past, present and future. Mol Cell Endocrinol 2001;178:117-31.

Shappell NW, Hyndman KM, Bartell SE, Schoenfuss HL. Comparative biological effects and potency of 17α- and 17β-estradiol in fathead minnows. Aquatic Toxicology 2010;100:1-8.

Shareef A, Angove M, Wells J, Johnson B. Sorption of bisphenol A, 17 alpha-ethynylestradiol and estrone to mineral surfaces. J Colloid Interface Sci 2006;297:62-9.

Sheng GD, Xu C, Xu L, Qiu Y, Zhou H. Abiotic oxidation of 17 beta-estradiol by soil manganese oxides. Environmental Pollution 2009;157:2710-5.

Snow DD, Damon-Powell T, Onanong S, Cassada DA. Sensitive and simplified analysis of natural and synthetic steroids in water and solids using on-line solid-phase extraction and microwave-assisted solvent extraction coupled to liquid chromatography tandem mass spectrometry atmospheric pressure photoionization. Analytical and Bioanalytical Chemistry 2013;405:1759-71.

Stumpe B, Marschner B. Dissolved organic carbon from sewage sludge and manure can affect estrogen sorption and mineralization in soils. Environmental pollution (Barking, Essex : 1987) 2010;158:148-54.

Stumpe B, Marschner B. Factors controlling the biodegradation of 17β-estradiol, estrone and 17α-ethinylestradiol in different natural soils. Chemosphere 2009;74:556-62.

Sun K, Jin J, Gao B, Zhang Z, Wang Z, Pan Z et al. Sorption of 17 alpha-ethinyl estradiol, bisphenol A and phenanthrene to different size fractions of soil and sediment. Chemosphere 2012;88:577-83.

Tang T, Shi T, Li D, Xia J, Hu Q, Cao Y. Adsorption Properties and Degradation Dynamics of Endocrine-Disrupting Chemical Levonorgestrel in Soils. J Agric Food Chem 2012;60:3999-4004.

Tetreault GR, Bennett CJ, Shires K, Knight B, Servos MR, McMaster ME. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquatic Toxicology 2011;104:278-90.

Williams R, Johnson A, Smith J, Kanda R. Steroid Estrogen Profiles along River Stretches Arising from Sewage Treatment Works Discharges. Environmental science & technology 2003;37:1744-1750.

Writer JH, Barber LB, Ryan JN, Bradley PM. Biodegradation and Attenuation of Steroidal Hormones and Alkylphenols by Stream Bioflims and Sediments. Environ Sci Technol 2011;45:4370-6.

Xuan R, Blassengale AA, Wang Q. Degradation of Estrogenic hormones in a silt loam soil. J Agric Food Chem 2008;56:9152-8.

Yamamoto H, Liljestrand H. The fate of estrogenic compounds in the aquatic environment: sorption onto organic colloids. Water Science and Technology 2003;47:77-84.

Yamamoto H, Liljestrand H, Shimizu Y, Morita M. Effects of physical-chemical characteristics on the sorption of selected endocrine disrnptors by dissolved organic matter surrogates. Environ Sci Technol 2003;37:2646-57.

Yildirim K, Uzuner A, Gulcuoglu EY. Biotransformation Of Some Steroids By Aspergillus terreus MRC 200365. Collect Czech Chem Commun 2010;75:665-73.

Ying G, Kookana R, Ru Y. Occurence and fate of hormone steroids in the environment. Environment International [Environ Int] 2002;28:545-551.

Ying GG, Kookana RS. Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. Environmental Toxicology and Chemistry 2005;24:2640-5.

Zhang Y, Krysl RG, Ali JM, Snow DD, Bartelt-Hunt SL, Kolok AS. Impact of Sediment on Agrichemical Fate and Bioavailability to Adult Female Fathead Minnows: A Field Study. Environ Sci Technol 2015;49:9037-9047.

Zucchi S, Castiglioni S, Fent K. Progesterone Alters Global Transcription Profiles at Environmental Concentrations in Brain and Ovary of Female Zebrafish (Danio rerio). Environ Sci Technol 2013;47:12548-56.

Zucchi S, Castiglioni S, Fent K. Progestins and Antiprogestins Affect Gene Expression in Early Development in Zebrafish (Danio rerio) at Environmental Concentrations. Environ Sci Technol 2012;46:5183-92.

Yu Z, Huang W. Competitive sorption between 17 alpha-ethinyl estradiol and naphthalene/phenanthrene by sediments. Environ Sci Technol 2005;39:4878-85.

Zhao L, Zhu L, Yang L, Liu Z, Zhang Y. Distribution and desorption of perfluorinated compounds in fractionated sediments. Chemosphere 2012;88:1390-7.

Zhou JL, Liu R, Wilding A, Hibberd A. Sorption of Selected Endocrine Disrupting Chemicals to Different Aquatic Colloids. Environ Sci Technol 2007;41:206-13.