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Publication Date

1999-08-01

G402
XU2-7
no. 882

Pharmaceutically-Active Compounds in Alternative Water Supplies

By

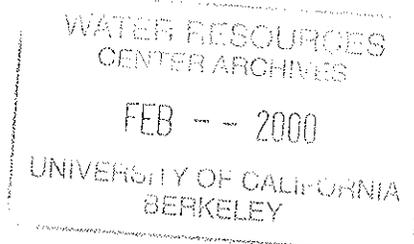
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TECHNICAL COMPLETION REPORT

Project Number UCAL-WRC-W-882

August, 1999

University of California Water Resources Center



The research leading to this report was supported by the University of California Water Resources Center, as part of Water Resources Center Project UCAL-WRC-W-882.

ABSTRACT

Pharmaceutically active compounds (PhACs) are a group of compounds that include hormones, antibiotics and painkillers. Results of several recent studies suggest that these compounds are present in wastewater effluents and in polluted surface waters. The possible presence of these compounds is especially significant to populated arid regions where wastewater effluents are used as drinking water and to augment freshwater habitats. To assess implications of PhACs in polluted waters, we have studied the fate and transport of several hormones. In the initial phase of the investigation, new analytical techniques were developed that are capable of detecting the hormones 17 β -estradiol, ethinyl estradiol and testosterone under the conditions encountered in polluted natural waters. Analysis of hormones in wastewater effluents from conventional treatment plants indicates that estrogenic hormones are present at concentrations comparable to those that cause endocrine disruption in fish under laboratory conditions. Testosterone is also present at similar concentrations, but no data are available on the threshold concentrations for endocrine disruption in fish. Hormones were detected at slightly lower concentrations in surface water samples, indicating that some removal may occur. Laboratory studies of hormone partitioning suggest that sorption is a relatively unimportant removal mechanism in surface waters. However, most hormones should be removed by adsorption during wastewater treatment. Additional research is needed to assess the mechanism through which hormones pass through wastewater treatment plants as well as their transformation in the aquatic environment.

KEY WORDS: effluents, hormones, wastewater, water quality, water reclamation, water reuse

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PROBLEM AND RESEARCH OBJECTIVES

As the population of California continues to grow, municipalities will be forced both to adopt stringent water conservation programs and to develop alternative sources of drinking water. In addition, new sources of water will be required to support or augment freshwater habitat. Since most pristine drinking water sources are already being utilized, future water supplies will likely include wastewater effluent and surface waters that have been subjected to agricultural and industrial pollution. As a result of public health and ecological concerns associated with alternative water sources, it is prudent to evaluate potential problems prior to investing in infrastructure to support new water supplies.

The degree of public interest in the potential risks of alternative water supplies is exemplified by the controversy surrounding recent water reuse projects. To address public health concerns, local municipalities have funded numerous studies to determine the concentrations of organic micropollutants, pathogens and disinfection byproduct precursors in recycled water (NAS, 1998). Although these studies suggest that extensive treatment of wastewater effluent results in safe drinking water, concerns have been raised about health related issues that have not yet been addressed. As a result of these concerns, the National Academy of Sciences (1998) recently concluded that planned reuse of wastewater effluent should be “an option of last resort”. To assure the safe and efficient development of new water supplies, the scientific community must anticipate potential public health threats and, if necessary, estimate the costs associated with different treatment technologies.

One potentially important public health issue that has not yet been addressed is the possible presence of pharmaceutically-active compounds (PhACs) in alternative water supplies. This class of compounds includes substances that are widely used in agriculture, medicine and biotechnology, such as antibiotics, drugs and hormones. Consideration of the use patterns of these compounds suggests that PhACs will be present in many alternative water sources. One reason why some PhACs are likely to be present in these waters is that they are not metabolized to a significant degree before they are excreted. In addition, they are unlikely to be removed during their passage through engineered or natural systems because many of them are relatively hydrophilic (*i.e.*, they are not strongly adsorbed by surfaces) and are resistant to chemical or biological degradation. Finally, unlike most potentially toxic substances that are produced in large quantities, the use and disposal of PhACs are virtually unregulated.

Several recent studies conducted in Europe have demonstrated that PhACs are present in wastewater effluent, polluted surface waters and drinking waters. Many of the detected compounds are common drugs (*e.g.*, painkillers, antibiotics and beta-blockers) that are prescribed in large quantities. For example, Ternes (1998) reported the presence of thirty-two PhACs in effluent samples collected from a wastewater treatment plant near Frankfurt, Germany. In nearby streams, many of the same PhACs were detected, but at lower concentrations. Other PhACs have been reported in wastewater and drinking water in Berlin (Herberer and Stan, 1996) and Switzerland (Buser *et al.*, 1998). Although little is known about the health effects of exposure to low concentrations of PhACs, the presence of these compounds in alternative water supplies is a significant concern.

In addition to the PhACs discussed above, several studies have documented the presence of estrogenic hormones in wastewater effluents. Estrogenic hormones are used in pharmaceuticals (*e.g.*, ethinyl estradiol in birth control pills and dihydroequilin in hormone replacement therapy) and are produced by the endocrine systems of vertebrates (*e.g.*, 17 β -estradiol). Fish are extremely sensitive to estrogenic hormones: concentrations as low as several parts-per-trillion (*i.e.*, ng/L) can adversely affect development and reproduction. This effect, which is known as endocrine disruption, is not limited to estrogenic hormones. Chemicals that interfere with other hormone systems (*e.g.*, androgen and thyroid axes) also can have adverse effects on aquatic organisms.

During the last several years, the presence of PhACs in alternative water supplies has become a significant concern to water suppliers in the United States. For example, the possible presence of PhACs in reclaimed water has affected the implementation of water reuse projects in Livermore, CA and San Diego, CA. As a result, research on the fate and transport of PhACs in the aquatic environment is a very high priority. Studies of the effects of PhACs also are necessary, because current water quality criteria do not address important endpoints such as endocrine disruption.

The objective of this project is to assess the fate and transport of one group of PhACs (*i.e.*, hormones) in alternative water supplies. After studying these compounds, additional studies can be designed to assess other classes of PhACs. To assess these compounds, research has been conducted in several phases. Topics studied include:

- Development and testing of analytical methods for quantifying hormones in polluted waters;

- Measurement of the concentrations of hormones in alternative water supplies and in wastewater effluent samples;
- Laboratory studies of the partitioning of hormones onto particles.

Although many issues must still be addressed before the public health threats posed by PhACs are fully understood, this two-year project has provided data that enhances our understanding of the problem.

METHODOLOGY

To assess the fate and transport of hormones in alternative water supplies, three different hormones were studied. The two estrogenic hormones (*i.e.*, 17β -estradiol and 17α -ethinyl estradiol) were chosen because they have been implicated in endocrine disruption in aquatic organisms (Desbrow *et al.*, 1998). 17β -estradiol is a potent natural estrogen produced by humans while 17α -ethinyl estradiol is the most common estrogen used in birth control pills.

Testosterone was chosen because it is a potent human androgen.

Unless otherwise specified, all chemicals were obtained from Fisher Scientific at the highest possible purity. Hormones were purchased from Sigma (St. Louis, MO). Aqueous solutions were prepared in deionized water produced by a Nanopure system (Barnsted, Dubuque, IA).

Wastewater effluent samples were collected from three treatment plants located in California. Surface water samples were collected from the Santa Ana River (near the Riverside wastewater treatment plant and below the Prado wetlands) and the Colorado River (near the

intake point for San Diego's water supply). All samples were stored in PFE-lined bottles at 5°C until extraction and analysis. Most samples were extracted and analyzed within 5 hours of collection.

Hormones were extracted from 0.22 µm filtered wastewater samples using C-18 solid phase extraction discs (Empore, 3M Corporation, Minneapolis, MN). For wastewater effluent samples, 47 mm discs were used. The 47 mm extraction discs were conditioned prior to use by washing three times with 10 mL-aliquots of methanol followed by three washes with 10 mL-aliquots of water. Between 300 and 1,250 mL of wastewater effluent sample was extracted at a flow rate of 0.5 to 5 mL/min. For surface water samples, larger volumes of water (*i.e.*, approximately 4 L) were passed through 90 mm extraction discs using a pressurized filtration system (Millipore Corporation, Bedford, MA). The 90 mm extraction discs were conditioned prior to use by washing twice times with 200 mL-aliquots of methanol followed by two washes with 200 mL-aliquots of water. Between 1 and 4 L of sample was extracted at a flow rate of 400 mL/min.

After extraction, hormones were eluted by washing the extraction discs with methanol. The methanolic extracts were transferred from a Pyrex collection tube into 25-mL Pyrex volumetric flasks prior to drying under a gentle stream of nitrogen. The dried extracts were resuspended in 600 µL of water.

Some samples were subjected to enzymatic hydrolysis to convert glucuronide and sulfate conjugates back into active hormones. Hydrolysis was performed using glucuronidase enzyme (Sigma Scientific, Type H-1 isolated from *Helix pomatia*), which was prepared at a concentration

of 1.9 g/L in 0.1 M acetic acid buffer adjusted to pH 5.0 with 0.1 N NaOH. Some experiments also were performed using type L-2 glucuronidase enzyme. The final concentration of enzyme in corresponded to 800 units/mL. The enzyme was stored at -5°C and a stock solution was prepared immediately before each experiment. Hydrolysis was allowed to proceed for 16-20 hours at 35°C. The activity of the enzyme was confirmed in every experiment using a stock solution of 3,000 ng/L 17β-estradiol 17-glucuronide.

Hydrolyzed and unhydrolyzed samples were subjected to high-pressure liquid chromatography (HPLC) to remove interfering organic compounds. A 250 mm Alltima C-18 column with 5 μm particles, 9% carbon load (Alltech, Deerfield, IL) was used with a mobile phase consisting of 63% methanol/37% water at a flow rate of 1 mL/min. Between 90 and 200 μL of sample was injected. The hormones typically eluted between 16 and 18 minutes. Data on sample UV absorbance between 200 and 320 nm was collected using a diode array detector.

To evaluate the retention time of the estrogenic hormones, 1 mg/L standards of 17β-estradiol, 17α-ethinyl estradiol and testosterone were injected before and after wastewater effluent samples were separated. Retention times were determined by UV/visible detection. To prevent the possibility of sample contamination from the high concentration standard, the autosampler needle was washed with water twice after the high concentration standards were analyzed. ELISA analysis of deionized water samples fractionated immediately after the concentrated standard was injected indicated that carryover of the hormones did not occur.

HPLC fractions were dried at room temperature under a vacuum prior to analysis by enzyme-linked immunosorbent assay (ELISA). Samples were suspended in 300 μL of water and

analyzed using commercially available ELISA kits (Neogen, Lexington, KY). After adding the enzyme conjugates and chromophores supplied by the manufacturer, absorbance was measured with an automated microplate reader.

To evaluate the accuracy and precision of the method, duplicate and spike recovery samples were analyzed with each batch of samples. Recoveries were measured in two ways: (1) 3-10 ng/L of each hormone was added to three different aliquots of deionized water prior to extraction; (2) 3-10 ng/L of each hormone was added to three different wastewater effluent samples prior to extraction. To calculate recoveries in wastewater effluents, concentrations of hormones and their conjugates measured in the same samples were subtracted from concentrations measured in the recovery studies. Recovery experiments also were performed using the hormone conjugates. For 17β -estradiol sulfate, it was also necessary account for losses during enzymatic hydrolysis because only 30% of the compound is converted into 17β -estradiol by the enzyme. Duplicate analyses (*i.e.*, completely separate extractions and analyses of the same sample) were performed on approximately 60% of the samples.

Studies of the partitioning of hormones were conducted using soil and activated sludge. Soil samples, collected from a site in Berkeley, CA, were homogenized and sieved through a 0.35 mm screen. The organic matter content was 13%. Activated sludge samples were collected from a municipal wastewater treatment plant in Dublin, CA. Unsettled secondary wastewater samples were centrifuged to concentrate the activated sludge. Activated sludge was stored at 5° C prior to analysis.

Partitioning studies were conducted by suspending soil or activated sludge in 100 mL of tap water or deionized water (total mass of suspended solids 0.01-4 g/L). A small aliquot of 17 β -estradiol (*i.e.*, typically 1 mL) from a concentrated methanolic stock solution was added to the solution. The initial concentration of 17 β -estradiol ranged from 4 μ g/L to 1 mg/L. The bottle was placed on a shaker table for 0 to 72 hours. Samples were settled and, in some cases, filtered prior to analysis. Samples containing high concentrations of 17 β -estradiol (*i.e.*, 1 mg/L) were analyzed using UV/Visible spectroscopy. Samples containing lower concentrations were analyzed using ELISA.

To assess the possibility that hormones were associated with colloids, experiments also were conducted to assess the phase partitioning of hormones that passed through 0.22 μ m filters. A dialysis membrane (3,000 Dalton cutoff, Spectra/Por) filled with deionized water was equilibrated for 48 hours inside of a glass container filled with samples containing 17 β -estradiol. Samples collected from both sides of the membrane were analyzed by ELISA after equilibration.

PRINCIPLE FINDINGS AND SIGNIFICANCE

After a series of pre-concentration and cleanup steps, immunochemical methods were capable of detecting hormones in surface waters and wastewater effluents. The techniques used in this project were capable of quantifying the extremely low concentrations of hormones expected in wastewaters and surface waters (*e.g.*, <5 ng/L). Although confirmatory analysis by other techniques (*e.g.*, GC/MS/MS) is needed, the immunochemical technique is an efficient and accurate method for quantifying hormones.

Results of recovery experiments, in which 3-10 ng/L of hormones were added to filtered samples yielded average recoveries of approximately 75% (Table 1). Recoveries for the relatively polar hormone conjugate 17 β -estradiol sulfate suggest that some losses may occur as a result of incomplete extraction of the conjugates. Recoveries did not depend upon the nature of the treatment processes or the organic carbon content of the samples. Experiments performed to analyze losses during HPLC fractionation and blowdown steps indicate that losses during these steps were minimal.

The detection limit of the immunochemical technique depends upon the matrix. In secondary wastewater effluent, a detection limit of approximately 0.1 ng/L was obtained for the hormones. Further pre-concentration of the wastewater effluent did not increase the sensitivity of the method because organic matter interferes with the immunoassay, leading to elevated background signal. In surface water samples, a detection limit of approximately 0.02 ng/L was obtained for the hormones through the use of a higher pre-concentration factor.

Results from ELISA analyses indicate that hormones are present at concentrations as high as approximately 4 ng/L in wastewater effluents (Table 1). Concentrations detected in surface waters are generally lower than those detected in wastewater. (Analyses of some samples were performed as part of a related project funded by the National Water Research Institute and are included here for comparison.) For wastewater effluents, the concentrations of hormones were related to the sophistication of the treatment system. Concentrations of hormones were generally highest in the effluent of WWTP 1, a plant that employs only primary and secondary treatment. Lowest concentrations of hormones were observed in the effluent of WWTP 3, an

Table 1

Concentration of hormones in wastewaters and surface waters.

Estrogenic Hormones:		17 β -Estradiol:			Ethinyl Estradiol:			17 β -Estradiol Conjugates:				
Date	WWTP	pH	UV254	recov. %	conc. (ng/L)	std dev.	recov. %	conc. (ng/L)	std dev.	G-recov. %	S-recov. %	(ng/L)
12/9/97	WWTP 1	7.61	NA	71.9	4.18	0.28	NA	NA	NA	65.9	66.3	<0.1
1/27/98	WWTP 1	7.43	0.2531	92.6	3.26	NA	NA	NA	NA	62.8	64.7	0.28
3/11/98	WWTP 1	7.26	0.2280	81.0	4.57	0.21	73.8	2.28	0.25	44.4	50.7	<0.1
6/10/99	WWTP 1	7.32	0.2224	68.1	3.68	0.86	NA	2.42	0.60	NA	NA	NA
	average=	7.41	0.235	78.4	3.92			2.35		57.7	60.5	
	std dev.	0.15	0.02	10.9	0.57			0.10		11.6	8.6	
6/19/98	WWTP 2	7.28	0.1560	48.6	1.23	0.17	70.6	0.42	0.03	140.6	81.1	<0.1
7/23/98	WWTP 2	7.06	0.1774	116.5	0.47	0.20	109.9	0.30	0.19	89.1	NA	<0.1
9/28/98	WWTP 2	7.05	0.1634	61.7	1.13	0.29	74.6	0.55	0.15	116.6	73.0	<0.1
	average=	7.13	0.17	75.6	0.94		85.0	0.43		115.4	77.1	
	std dev.	0.13	0.01	36.1	0.41		21.6	0.12		25.8	5.7	
3/31/98	WWTP 3-MF	NA	NA	50.7	2.65	NA	75.9	0.16	NA	NA	NA	<0.1
12/14/98	WWTP 3-MF	6.91	0.1738	92.2	0.72	0.14	NA	<0.1	0.03	68.5	NA	<0.1
6/10/99	WWTP 1-MF	7.49	0.2014	NA	NA	NA	NA	NA	NA	NA	NA	NA
	average=	7.20	0.1676	71.5	1.68		75.9			68.5		
	std dev.	0.41	0.02	29.3	1.36							
3/31/98	WWTP 3-RO	NA	NA	63.3	<0.1	NA	50.9	<0.1	NA	NA	NA	0.13
12/14/98	WWTP 3-RO	5.67	0.0546	62.5	0.32	0.00	82.4	<0.1	0.02	146.4	NA	<0.1
6/10/99	WWTP 1-RO	NA	NA	35.8	0.24	0.11	31.9	<0.1	0.02	NA	NA	NA
	average=	5.67	0.055	53.9	0.28		55.1			146.4		
	std dev.			15.7	0.06		25.5					

Androgenic Hormones:		17 β -Estradiol:			Ethinyl Estradiol:			17 β -Estradiol Conjugates:				
Date	WWTP	pH	UV254	recov. %	conc. (ng/L)	std dev.	recov. %	conc. (ng/L)	std dev.	G-recov. %	S-recov. %	(ng/L)
12/15/98	Surface water -near Colorado River -near San Diego	7.76	0.1047	100.5	0.80	0.24	NA	<0.1	0.08	85.7	NA	<0.1
7/12/99	Surface water -Riverside -Prado	NA	NA	72.0	3.20	0.27	NA	NA	NA	NA	NA	NA
		NA	NA	111.0	0.37	NA						

Androgenic Hormones:

Date	WWTP	pH	UV254	Testosterone: recov. %	conc. (ng/L)	std dev.
7/7/99	WWTP 1	NA	NA	78.0	0.83	0.17

Notes:

Reported concentrations have been adjusted for recoveries. WWTP 1 is an activated sludge municipal wastewater treatment plant equipped with a pilot advanced wastewater treatment plant. Samples were collected after secondary treatment, after microfiltration and after reverse osmosis. NA: data not available.

advanced wastewater treatment plant. In surface water samples, hormone concentrations as high as 3.2 ng/L were detected in the Santa Ana River, proximate to the discharge point for the Riverside wastewater treatment plant. Concentrations below 1 ng/L were detected in other surface water samples.

Our measurements of estrogenic hormones in wastewater effluents are comparable to results from other studies. For example, our median concentration of 17 β -estradiol (*i.e.*, 1.2 ng/L) is comparable to the median concentrations determined by GC/MS/MS (*i.e.*, 0.7, <1 and 6 ng/L; Ternes *et al.*, 1999; Belfroid *et al.*, 1999) and GC/MS (*i.e.*, 5.9 ng/L; Desbrow *et al.*, 1998). The maximum concentration detected by immunoassay (*i.e.*, 4.6 ng/L) is lower than that determined by GC/MS or GC/MS/MS (*i.e.*, maximum concentrations reported in other studies are as high as 64 ng/L). The highest concentrations measured by conventional techniques are approximately two to three times higher than the estimated concentrations in wastewater influent. On the basis of hydrophobicity (*i.e.*, we estimate the octanol/water partition coefficient for 17 β -estradiol to be approximately 15,000) estrogenic hormones should have some affinity for particles in wastewater. For example, using the octanol/water partition coefficient to estimate the linear distribution coefficient, K_D (Schwarzenbach *et al.*, 1993), we estimate that approximately 70% of the 17 β -estradiol will be associated with particles during activated sludge treatment (assuming 2 g/L volatile suspended solids). Although simultaneous measurements with immunoassays and GC/MS/MS are needed, the fact that 17 β -estradiol concentrations measured with immunoassays were never more than approximately 25% of the expected influent concentrations suggests that the relatively high concentrations of hormones reported in some studies could be an artifact.

Our measurements of estrogenic hormones in surface waters also are comparable with previously published results. For example, Belfroid *et al.* (1999) reported concentrations of 17 β -estradiol ranging from <1 ng/L to 5.5 ng/L in surface waters collected in the Netherlands. However, in all previous studies hormones were only detected in a small fraction of the samples because the detection limits were significantly higher than those obtained with the immunochemical method. Therefore, the immunochemical technique offers significant advantages over conventional analytical techniques with respect to sensitivity, accuracy and effort.

The concentrations of estrogenic hormones detected in wastewater effluents could explain endocrine disruption observed in surface waters. For example, Routledge *et al.* (1998) reported the induction of endocrine disruption in Rainbow Trout at concentrations between 1 and 10 ng/L. Because all of the estrogenic hormones act on the same receptor, the effects of the mixture should be additive.

Detection of testosterone in wastewater effluents represent the first reports of this compound in the aquatic environment. The concentration detected in wastewater effluent is approximately equal to those of the estrogenic hormones. Although we are unaware of any studies linking exposure to testosterone to androgen disruption in fish, recent data on binding of testosterone to fish receptors suggest that testosterone has a high affinity for estrogen binding receptors in fish (Wells and van der Kraak, 1998).

Results of partitioning experiments indicate that the affinity of 17 β -estradiol for soil or activated sludge particles are consistent with predictions based on hydrophobic interactions. The measured linear distribution coefficient (K_D) was 310 L/kg and 10,400 L/kg, for soil and activated

sludge, respectively (see Figure 1 for example results). These differences are consistent with the higher organic matter content of activated sludge (*i.e.*, the activated sludge contained approximately ten times more organic matter by than the soil sample). Kinetic experiments conducted with activated sludge (Figure 2) indicate that partitioning occurs via a rapid adsorption followed by slow loss after approximately 4 hours. Further research is needed to assess the possibility that the slow loss is attributable to biotransformation.

Partitioning experiments conducted at low concentrations of estradiol (*i.e.*, 4 µg/L) suggested deviation from linear partitioning. To assess the possible association of 17β-estradiol with humic matter, preliminary experiments were with Aldrich humic acid. Although results were inconclusive, previous studies conducted with the hydrophobic steroid coprostanol (Hassett and Anderson, 1979) suggest that association with humic substances is possible. Additional studies with radiolabelled compounds are needed to assess this possibility.

Results of the simple partitioning experiments allow us to make a preliminary assessment of the importance of partitioning to the removal of hormones and other PhACs in engineered and natural systems (Figure 3). For example, 17β-estradiol and the other hormones studied have octanol/water partition coefficients of approximately 10^4 . Therefore, adsorption onto particles should be significant whenever particulate organic matter (POM) exceeds 10^{-4} kg/L (*i.e.*, 100 mg/L). Such conditions are encountered in wastewater treatment plants (*i.e.*, POM is approximately 10^{-3} kg/L in activated sludge treatment plants) but not in most surface waters. For many other PhACs, K_{ow} is much less than 10^2 . For such compounds, adsorption will be insignificant in most engineered and natural systems.

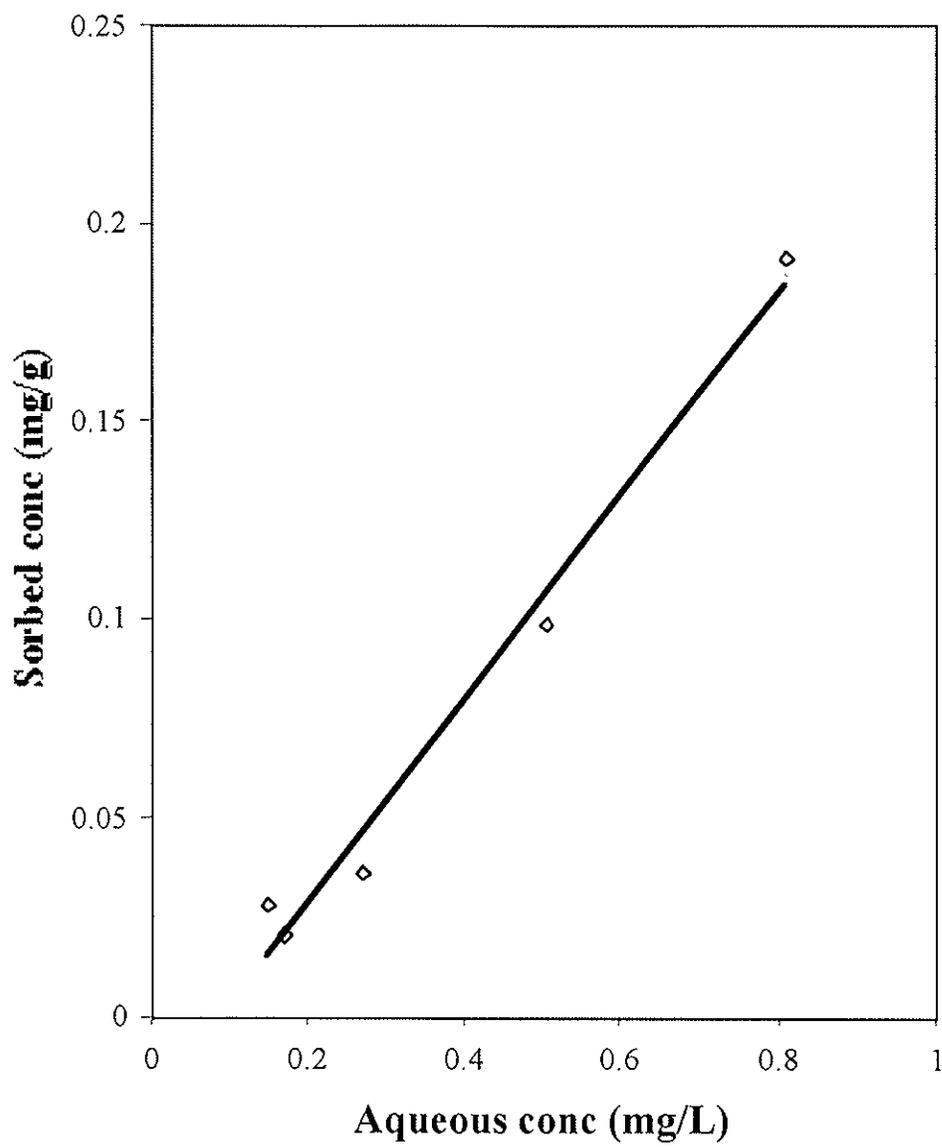


Figure 1: Adsorption isotherm for 17 β -estradiol on soil. Initial concentration = 1 mg/L.

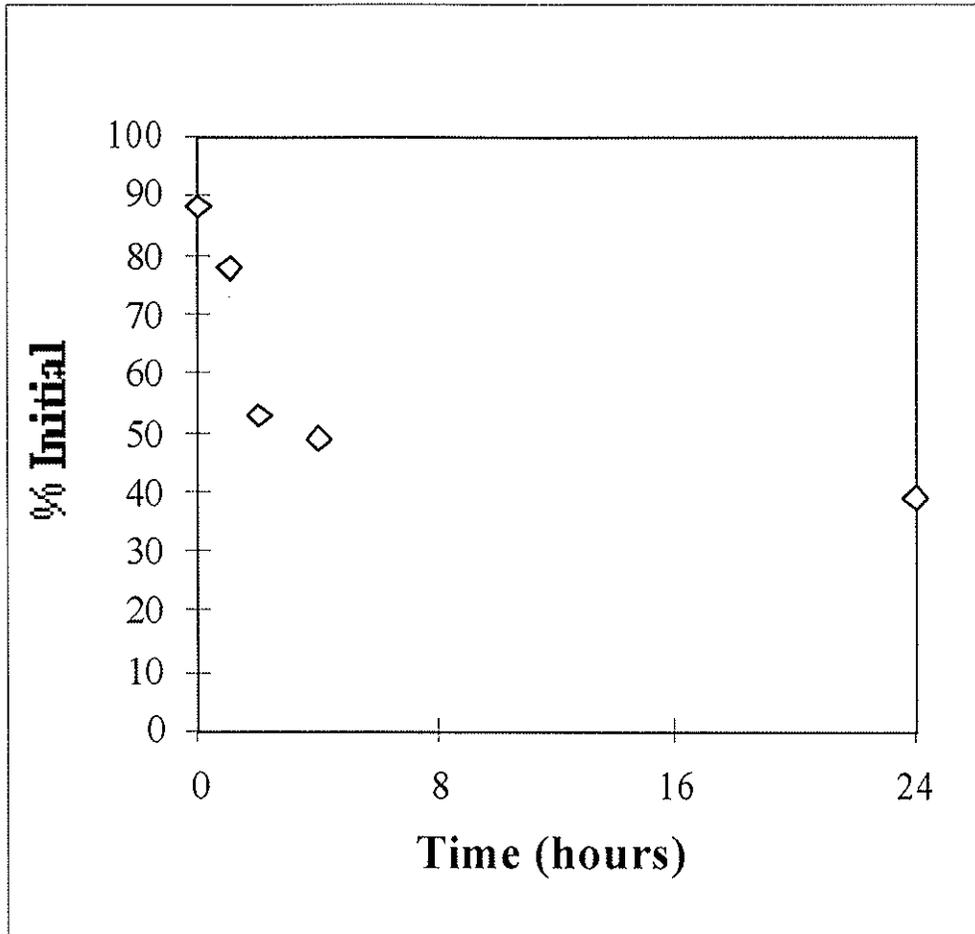


Figure 2. Removal of dissolved 17β -estradiol added to activated sludge samples. Initial estradiol concentration = $50 \mu\text{g/L}$.

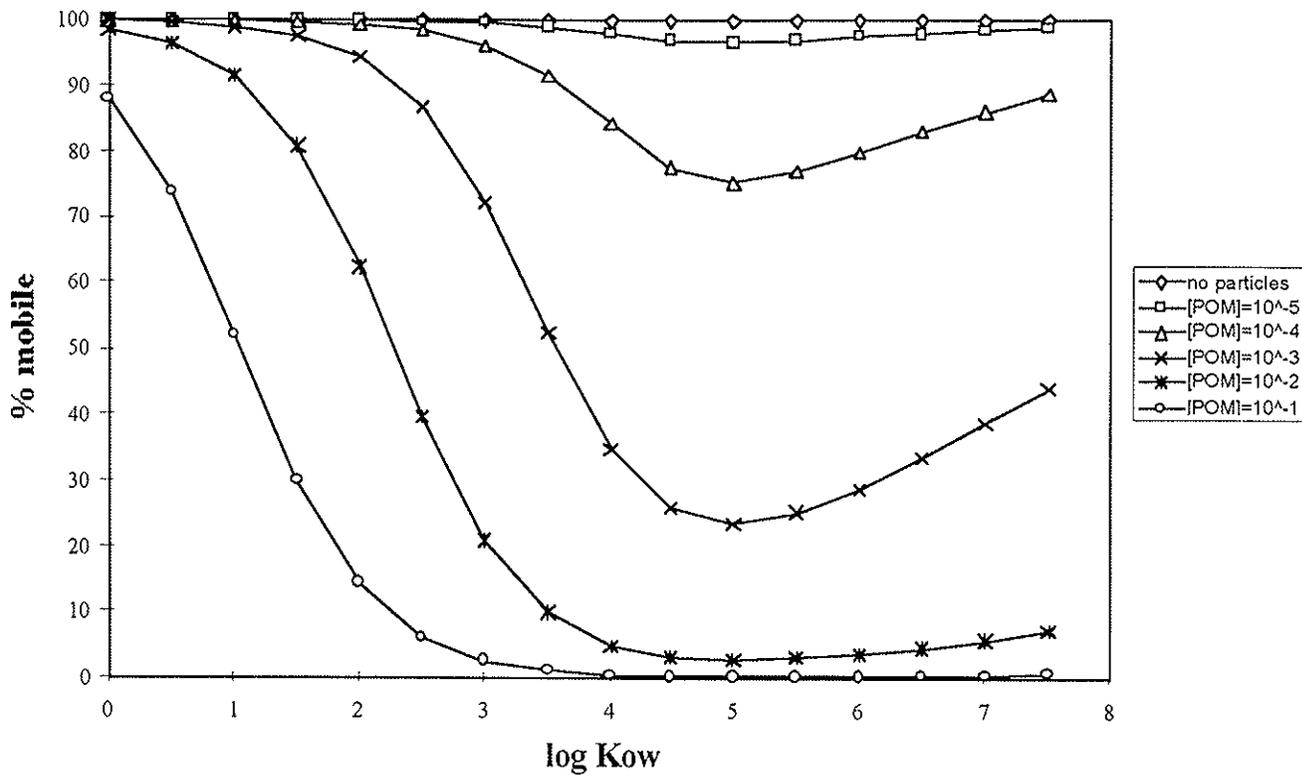


Figure 3: Predicted phase association of organic compounds exposed to particulate organic matter assuming linear partitioning to particulate and colloidal organic matter. % mobile refers to percent of total compound that cannot be removed by filtration. K_{ow} refers to the octanol/water partition coefficient.

Findings of this project indicate that hormones are present in wastewater effluents at concentrations capable of causing endocrine disruption in fish. The presence of relatively high concentrations of hormones in wastewater effluents is unexpected because the compounds should be removed by adsorption onto activated sludge in secondary treatment systems. The hormones are removed by advanced wastewater treatment systems and by natural attenuation in surface waters. Further research is needed to assess processes resulting in removal of hormones during wastewater treatment and their transformation in surface waters.

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