Report for 2001MO3002B: Identification and Biological Screening of Endocrine Disruptors in Effluents from Missouri Sewage Treatment Plants

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Identification and Biological Screening of Endocrine Disruptors in Effluents from Missouri Sewage Treatment Plants

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Effluent samples were taken from four Missouri sewage treatment plants (STP). Each effluent sample was subject to a solid phase extraction (SPE) and then analyzed for endogenous estrogens, synthetic estrogens, pesticides, herbicides, industrial chemicals, and α-zearalenol (a mycotoxin) using LC/MS and GC/MS. An aliquot of each sample from the SPE were reconstituted in DMSO, which were further diluted with medium for testing their ability to induce MCF-7 cell proliferation. The proliferation data from each reconstituted water extract were compared with 17β-estradiol (E2), a standard curve. Chemicals that were detected and/or have been reported to be estrogenic were also tested individually to calculate EC50 values, relative proliferative potency (RPP), and relative proliferative effect (RPE) in stimulating MCF7 cell proliferation (Figures 1 – 3).

The chemical detection limits are between 0.1 ng/L and 0.9 ng/L, whereas the recovery of the chemicals are between 51.7% and 93.3% (Table 1). Among the endogenous and synthetic estrogens, only 17α-ethinylestradiol was found once in the Columbia STP at 2.9 ng/L water (Tables 2 – 4). In general, industrial chemicals such as 4-octylphenol, nonylphenols, 4-tert-octylphenol, dibutyl phthalate, butyl benzyl phthalate, and bisphenol A are more prevalent in the effluents compared with the other types of chemicals (Tables 2 – 4). However, these industrial chemicals are less estrogenic than endogenous and synthetic estrogens (Table 5). Figure 1 shows that the effluent extracts from the Little Blue Valley, Kansas City, and Columbia STPs are capable of stimulating MCF7 cell proliferation. The induction can be inhibited by antiestrogen (i.e. tamoxifen) co-treatments (data not shown), indicating that the cell proliferation is mediated via an estrogen receptor (ER) signal transduction pathway. The estrogenic effects might be caused by industrial chemicals detected and/or other chemicals that were not identified in our chemical analysis. Whether the effluents would have imposed estrogenic effects on organisms inhabiting the STP downstream ecosystems remains to be investigated.

INTRODUCTION

Estrogen, an endogenous sex steroid hormone, plays a major role in secondary sex organ development, behavior, fertility, and reproductive capacity. Many environmental pollutants such as herbicides, pesticides, phytochemicals, industrial wastes, and pharmaceuticals have been reported to possess estrogen-like activity (*1*). It has been shown that exposure to these xenoestrogens causes abnormalities in reproductive organs and malformations in wildlife (*1-3*). For instance, studies in England (*4-6*) and the USA (*7*) have shown that male fish held in treated sewage effluents exhibit increased levels of vitellogenin, an indication of exposure to estrogenic chemicals.

Sewage treatment plants (STPs) receive influent from domestic, municipal, and industrial sewage systems. The estrogenic chemicals in the influent may include endogenous 17β-estradiol (E2) and its metabolites (i.e., estrone, estriol), synthetic estrogens such as 17α-ethinylestradiol, personal care products, and industrial discharges such as akylphenols. Pyrethroid insecticides and herbicides in domestic use may also end up in the sewage treatment plants. To date, the existence of estrogenic chemicals and their levels in the output of the metropolitan sewage treatment plants in Missouri are still

unsubstantiated. The potential impact of these chemicals on the quality of surface and ground waters, public health, and aquatic ecosystems remains to be elucidated. In this study we planned 1) to identify the estrogenic chemicals that may be present and determine their levels in Missouri STP effluents; and 2) to evaluate the total estrogenicity induced by effluent extracts from different STPs using the MCF7 cell proliferation test.

Effluents were collected from four sewage treatment plants in Missouri to identify and determine the levels of estrogenic chemicals. Sixteen chemicals that are endogenous estrogens, synthetic estrogens, herbicides, pesticides, and industrial chemicals were analyzed using liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). The water extracts were subject to the MCF7 cell proliferation test that has at least two merits (*8*). First, the reported detection limit of 10 pg /ml E2 makes MCF7 cell proliferation assay one of the most sensitive *in vitro* assays for assessing the estrogenicity of xenoestrogens. Second, there have been few reported cases of false positive results using the MCF7 E-Screen.

METHODS AND MATERIALS

17α-Ethinylestradiol (98%), estrone (99.3%), and estriol (99%) were purchased from ICN Biomedicals (Aurora, CA, USA). 17β-Estradiol (E2, ≥98%), β-estradiol-3-benzoate (EB, 98%), diethylstilbestrol (DES, ≥99%), and dimethyl sulfoxide (DMSO, >99.5%) were purchased from Sigma (St. Louis, MO, USA). α-Zearalenol (97%), dibutyl phthalate (DBP, >99%), bisphenol A (Bp A, >99%), and 4-tert-octylphenol (97%) were purchased from Aldrich (Milwaukee, WI, USA). Butyl benzyl phthalate (BBP, 98%), atrazine (98%), simazine (98%), fenvalerate (99%, cis), bioallethrin (d-trans-allethrin, cis: trans = 2 : 96), and permethrin (cis : trans = 20:78) were purchased from ChemService (West Chester, PA, USA). SPE cartridges (Part # 188-1360) containing 1g of C_{18} adsorbent each were purchased from J&W Scientific (Folsom, CA). The MCF7 breast cancer cell line was purchased from the Karmanos Cancer Institute (Detroit, MI). DMEM F12 medium, calf serum, and penicillin/streptomycin were purchased from Life Technologies (Grand Island, NY, USA). All other chemicals and reagents were of the highest quality available from commercial sources.

Sewage Treatment Plants and Effluent Sample Collection

Approximately 4 - 8 liters of representative 24-h composite samples of effluents from municipal sewage treatment plants in St. Louis (Bissell Plant), Columbia, Kansas City in Missouri (Blue River Plant), and Independence (Little Blue Valley Plant) were taken with an automatic, time-proportioned sampling device (Isco 3710, Lincoln, NE, USA) in June, August, and October 2001. All water samples were placed in pre-cleaned glass bottles and stored in the freezer (-20°C) until testing. These four plants have mechanical purification, active sludge treatment, biological nitrate removal, and settlement tanks as major cleaning processes.

Solid Phase Extraction

Within 2 - 3 days after collection, organic materials were recovered from the water sample by a solid phase extraction (SPE) using octadecylsilane (C_{18}) coated supports. Five mL of methanol was added to 1 liter of water sample. The water sample

was then filtered through a vacuum filtration apparatus with a glass microfiber filter (Part # 1823 047, Whatman International, Maidstone, England). The filtered water sample was pulled through a conditioned SPE cartridge at a flow rate of 10-15 mL/min by adjusting the vacuum. The SPE cartridge was washed with 6mL of deionized water, and the SPE adsorbents were dried by pulling air for a while and storing in a desiccator. The dried SPE cartridge was connected to a disposable pipette filled with anhydrous sodium sulfate. The organic materials in the SPE cartridge were eluted into an empty graduated tube by passing 3 mL of acetone twice. The extract was concentrated to 1mL under gentle stream of nitrogen. Half of the extract was transferred to a tube for further chemical analysis. Fifty μL of DMSO was added to the remaining extract, and acetone was completely removed under a gentle stream of nitrogen. This fraction of water was stored at –70^{o}C for the subsequent cell proliferation assay.

Chemical Analysis

The extracted organics were analyzed for the presence of estrogenic chemicals such as steroidal estrogens, pesticides, herbicides, and industrial chemicals using LC/MS and GC/MS techniques.

1. Silica Gel Fractionation

Prior to LC/MS and GC/MS analyses, the SPE samples were fractionated with a silica gel column which contained 1 gram of silica gel 60 sorbent (70-230 mesh, Fisher Scientific, Pittsburgh, PA) deactivated with 1.5% water. Before adding the sample, the silica gel column was rinsed with 10 mL of hexane/acetone (60:40) mixture. After transferring SPE sample to the column, the analytes were eluted with 10 mL of hexane/acetone (60:40) mixture.

2. LC/MS Analysis

A liquid chromatography/mass spectrometry (LC/MS) analysis of the analytes was performed with Hitachi M-8000 LC/MS system. Compounds of interest were separated on a reversed phase LC column (Xper-Chrom, 4.6 mm X 25 cm, P.J. Cobert Associates, Inc., St. Louis, MO) packed with C_{18} coated 5 μ supports. A 35-minute gradient program of 100% to 0% water using two mobile phases (water and acetonitrile with 1% acetic acid additive) was used for the separation of the analytes. The effluent from the column was split and sent to both MS and diode array detectors. The diode array detector wavelength was set at 220 nm. The ion trap mass spectrometer is operated in positive and negative modes with an electrospray ionization source.

3. GC/MS Analysis

A gas chromatography/mass spectrometry (GC/MS) analysis of the SPE sample was performed using a capillary column (30m x 0.25mm i.d.) coated with 5% phenyl – 95% methyl polysiloxane stationary phase (DB-5, J&W Scientific, Folsom, CA). A temperature program was started from 120 °C and increased to 190 °C at 10 °C/min rate and further increased to 300 °C at 5 °C/min. A Hewlett-Packard 5971 GC/MS system was operated in both scan (45-550 amu) and selected ion monitoring modes for identification and quantitation of the analytes.

The quality of all analytical procedures was controlled by inclusion of validation samples with reference spikes, replicates, and blanks. The blank was demineralized water to check whether the clean-up step introduce estrogenic contaminants into the water samples. The blank was also used in the cell proliferation to test its estrogenicity. Table 1 showed that the detection limits ranged from 0.1 ng/L to 0.9 ng/L, whereas the recovery percentage ranged from 51.7% to 93.3%.

Subsequently, the organic materials recovered from the solid phase extraction were assayed using the MCF7 cell proliferation test.

Preparation of Water Extract for the Cell Proliferation Test

4.95 ml steroid-free experimental medium were added to each water extract, votexed, and the solution was sterile filtered through a 0.22 μm membrane. These stock solutions containing 1% (v/v) DMSO further diluted to 10- to 5,000 fold (0.05 – 25 L final volume) with steroid-free experimental medium using sterile 50 ml polypropylene tubes. The maximum DMSO or ethanol concentrations in the final medium were kept at 0.1% for every dilution, a concentration which did not affect cell yield (*9*).

MCF7 Cell Proliferation Assay

This assay used the human ER-positive MCF7 breast cancer cell line (Karmanos Cancer Institute, Detroit, MI) to quantitatively determine the total estrogenic activity of the above STP water extracts. The protocol in this present study was adapted from other publications (*8-10*) and described as follows.

Cells were maintained in DMEM F12 medium supplemented with 10% calf serum and 100 IU/ml penicillin, and grown at 37^{o}C in a 4% CO_2 humidified environment. Cells were inoculated into 12-well plates at a density of 6,000 cells per well and allowed to attach for 24 h. After 24 hours, the medium was aspirated and replaced with 1 mL experimental medium treated with dextran-coated charcoal to remove all steroids. Single chemicals were prepared in either DMSO or ethanol, depending on their solubility in these two solvents. Single chemicals and STP effluents were tested in a series dilution containing 6 to 8 concentrations, with each dilution tested in triplicate per assay. Three wells containing appropriate solvent but without test chemicals were used as negative control. 17β-Estradiol (in either DMSO or ethanol) between 10^{-14} M and 10^{-8} M were used as positive control in each assay. Concentrations ranging from 10^{-13} M and 10^{-4} M were used to test individual chemicals that have been reported to be estrogenic and or commonly present in STP effluents or in environmental samples. Additionally, a fixed ratio dilution of each effluent sample was tested together with 5 μM of the antiestrogen tamoxifen to validate that the induction of cell proliferation was mediated by an ER-mediated signal transduction pathway. Each single chemical or effluent was tested at least three times.

Six days after exposure, the assay was terminated during the late exponential phase of proliferation by determination of the cell numbers in each well using the sulforhodamine B assay (*10, 11*). The experimental medium was discarded, the 12-well plates were washed with 500 μl cold phosphate buffered saline per well, and then fixed for at least 40 min with 200 μl cold 10% (w/v) trichloriacetic acid (TCA). After TCA was discarded, the cells were washed three times with tap water and dried completely

under a hood at the room temperature. Staining of the cells was performed by adding 250 µl solution of 0.2% sulforhodamine B in a 1% acetic acid to each well. After 20 minutes, the staining solution was discarded and the cell were washed several times with 1 % acetic acid until the washing solution was colorless. After complete drying, the dye was dissolved in 300 µl cold 10 mM Tris buffer (pH 10.5) per well and extinction at 550 nm (reference 630 nM) was measured in triplicate per well with a microplate reader (FLOURstar, BMG Labtechnologies, Durham, NC, USA) by transferring aliquots of 100 µl into wells of 96-well plates.

Quantitative Evaluation and Statistics

Each chemical was tested at least three times. The data from the cell proliferation assay were fitted into a sigmoidal dose response equation with a variable slope (a.k.a. four-parameter logistic equation). EC50 was defined as the concentration of a chemical that induced cell proliferation half way between the baseline (i.e., bottom) and the maximal level (i.e., top). The equation was expressed as follows and the EC50 value was then derived from it.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1+10^{(\log EC50 - X)*\text{HillSlope}}}$$

We adjusted the EC50 of a chemical with the EC50 of E2 to indicate its relative proliferative potency (RPP), and it was calculated as follows.

$$RPP = EC_{50}[E2] / EC_{50}[\text{test chemical or effluent}]$$

In addition to the EC50 values and its comparison against E2, fold induction was also taken into consideration. The relative proliferative effect (RPE) indicated the relative proliferative response of a specific chemical compared with that of E2. Thus if a chemical was a full agonist, its RPE was 100. If a chemical was a partial agonist, its RPE was below 100. The RPE was determined as follows.

RPE = (Maxi induction fold of a chemical or an effluent) / (Maxi. induction fold of E2)

The probit regression and calculation of EC_{50} values was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

RESULTS & DISCUSSIONS

Chemical Data

Twenty-four hour composite water effluents from the STPs in St. Louis, Columbia, Independence, and Kansas City (in Missouri) were collected on June 27-28, August 8-9, and October 18-19. The samples were subjected to LC/MS and GC/MS analyses, and the chemical data were shown in Tables 2 – 4. Endogenous and synthetic

estrogens were below detection limits, except for 17α-ethinylestradiol which was found only once at a concentration of 2.9 ng/L water in the Columbia STP effluent (Table 4). Permethrin and bioallethrin were not found. Atrazine was detected at low single digit ng/L levels in all of the STPs, whereas simazine was found only once at 2.3 ng/L water in the Independence STP (Table 3). 4-Octylphenol and 4-tert-octylphenol were either below the detection limits or at less than 10 ng/L water. Compared with 4-octylphenol and 4-tert-octylphenol, nonylphenols were present in all of the plants during our survey, and could be as high as 226.1 ng/L water (Table 4). The levels of dibutyl phthalate were between 2.9 ng/L water and 5.1 ng/L water in the first sampling trip (Table 2), whereas its levels were higher between 3.5 ng/L water and 35.2 ng/L water in the two subsequent sampling events (Tables 3 – 4). Butyl benzyl phthalate was detected in all of the plants at the lower single digit ng/L levels in each sampling event. Bisphenol A was detected twice in the Columbia plant at 18.4 ng/L and 68.5 ng/L, and was not found in the other three plants. Overall, the Columbia plants tend to have higher concentrations of industrial chemicals. In general, industrial chemicals (i.e., 4-octylphenol, nonylphenols, 4-tert-octylphenol, dibutyl phthalate, butyl benzyl phthalate, and bisphenol A) were more prevalent and had higher levels in the effluents compared with the endogenous estrogens, synthetic estrogens, pesticides, and herbicides.

Biological Data

The estrogenic responses of MCF7 cells to single chemicals tested were shown in Table 5. The average maximal induction folds of 17β-estradiol (E2 in EtOH), estriol, 17α-ethinylestradiol, β-estradiol benzoate (β-EB), and diethylstilbestrol (DES) were between 5 – 6 folds, whereas estrone was at 3.91 fold (Figure 1 & Table 5). E2, estriol, 17α-ethinylestradiol, β-EB, and DES showed similar relative proliferative effects (RPE). On the other hand, the RPE value of estrone was approximately 24% lower than those of E2 and others.

Though in general the above endogenous and synthetic estrogens possessed similar maximal induction folds and RPE values, their EC50 values and relative proliferative potencies (RPP) showed significant discrepancy ranging from 0.02 to 0.94, with 17α-ethinylestradiol the highest and estrone the lowest (Table 5). These data indicated the importance of taking both RPE and RPP into consideration when estrogenicity was compared among chemicals. The EC50 and RPE values of mycotoxin, α-zearalenol, were similar to those of estriol. In summary, the above chemicals can be classified as strong environmental estrogens as they were able to illicit high levels of cell proliferation.

Toxicity was observed at 10^{-4} M in 4-*tert*-octylphenol, nonylphenol, and Bisphenol A. At this concentration the cells in the wells were completely lost. Due to toxicity at 10^{-4} M, these three chemicals showed incomplete sigmoidal growth curves. Thus their EC50 values and average maximal induction folds were calculated based on the assumption that 10^{-5} M of E2 stimulated the highest induction of cell proliferation (Figure 2 & Table 5). Nonylphenols induced 3.94-fold and 3.19-fold cell proliferation at 10^{-6} M and 10^{-5} M, respectively, with an EC50 value of 0.45 ± 0.61 μM, which was about 10^{5}-fold higher than that of E2. Bisphenol A induced approximately 3.8-fold and 3.4-fold cell proliferation at 10^{-6} M and 10^{-5} M, respectively, with an EC50 value of 0.28 ± 0.05 μM. 4-*tert*-Octylphenol induced 4.2-fold and 3.6-fold cell proliferation at 10^{-6} M

and 10^{-5} M, respectively, with an EC50 value of 0.31 ± 0.15 M. Permethrin induced 2.97-fold cell proliferation and the EC50 value was 1.66 ± 1.46 μM (Figure 3 & Table 5). One difference between permethrin and the above three chemicals was that the cells treated with permethrin at 10^{-4} M showed a similar growth rate as those of the control group, indicating some degree of cytotoxicity at this concentration. Overall, these four chemicals showed similar estrogenicity based on their similar RPP and RPE values, though bisphenol A had a bit higher RPE and a lower RPP. In summary, compared to E2, these four industrial chemicals were considered as weak environmental estrogens.

On the other hand, bioallethrin, fenvalerate, atrazine, simazine, and dibutyl benzyl phthalate at up to 10^{-5} M did not induce MCF7 cell proliferation (figures not shown). Toxicity was observed at 10^{-4} M of these chemicals. Because these five chemicals did not induce MCF-7 cell proliferation, they were classified as non-estrogenic chemicals.

The STP effluent samples collected on October 18-19 were solid phase extracted, and then diluted to test twice for their estrogenicity in MCF-7 cell proliferation. The St. Louis sample was lost during the sample filtration because of the problem with the vacuum system. The maximal cell proliferation induction by the Little Blue Valley and Kansas City STP effluents were approximately 2.85- and 3.05-fold, respectively, and were as high as that of E2, 3.18-fold (Figure 4). The maximal cell proliferation induction by the Columbia STP effluent was at 2.86-fold. All three effluents showed their highest induction at either 0.5-L or 1.25-L equivalent dilution. At 0.05-L equivalent dilution, the cell growth rates of these three effluents were approximately 1- to 1.5-fold of the control group, indicating possible cytotoxicity that might have reduced the growth rates. The induction could be inhibited by antiestrogen (tamoxifen) co-treatments (data not shown), indicating that the cell proliferation was mediated via an estrogen receptor-mediated signal transduction pathway. We suspected that the estrogenic effects might be caused by industrial chemicals and other chemicals that were not identified in our chemical analysis.

One technical limitation in relation to the cell proliferation test was that chemicals adsorbed in the solid phase of the effluent might be discounted. Moreover, though the MCF-7 cell proliferation by the effluents was observed, future studies may investigate whether the effluents would have imposed adverse ecological effects in the downstream ecosystems due to a significant dilution in the Missouri River.

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Table 1. Quality control for the chemical analysis of the STP effluents. Detection limit was determined based on 8-liter water samples. Concentrations were expressed as ng/L. R. Blank = Reagent Blank; R. Spike = Reagent Spike; DL = detection limit.

Chemical	Det. Limit	Mean R. Blank	Mean R. Spike % Recovery
17β-Estradiol	0.5	< DL	66.7
Estrone	0.5	< DL	70.8
Estriol	0.9	< DL	51.7
17α-Ethinylestradiol	0.5	< DL	79.2
β-Estradiol Benzoate	0.4	< DL	80.8
Diethylstilbestrol	0.8	< DL	72.5
Permethrin	0.5	< DL	70.0
Bioallethrin	0.3	< DL	84.2
Fenvalerate*	---	---	---
Atrazine	0.2	< DL	80.8
Simazine	0.3	< DL	77.5
4-Octylphenol	0.1	< DL	82.5
Nonylphenols	0.5	< DL	82.5
4-tert-Octylphenol	0.2	< DL	82.5
Dibutyl phthalate	0.1	1.2	80.8
Butyl benzyl phthalate	0.1	0.8	93.3
Bisphenol A	0.7	< DL	81.7
α-Zearalenol*	---	---	---

* Not analyzed, only tested for cell proliferation.

Table 2. Occurrence and levels of chemicals in Missouri sewage treatment plants on June 27-28 2001. Concentrations were expressed as ng/L. DL = detection limit.

Chemical	St. Louis	Columbia	Independence	Kansas City
17β-Estradiol	< DL	< DL	< DL	< DL
Estrone	< DL	< DL	< DL	< DL
Estriol	< DL	< DL	< DL	< DL
17α-Ethinylestradiol	< DL	< DL	< DL	< DL
β-Estradiol benzoate	< DL	< DL	< DL	< DL
Diethylstilbestrol	< DL	< DL	< DL	< DL
Permethrin	< DL	< DL	< DL	< DL
Bioallethrin	< DL	< DL	< DL	< DL
Fenvalerate*	---	---	---	---
Atrazine	2.9	< DL	< DL	< DL
Simazine	< DL	< DL	< DL	< DL
4-Octylphenol	< DL	< DL	1.8	2.1
Nonylphenols	4.5	5.1	18.1	43.3
4-tert-Octylphenol	< DL	< DL	< DL	< DL
Dibutyl phthalate	2.9	3.5	3.5	5.1
Butyl benzyl phthalate	1.4	1.3	3.5	3.6
Bisphenol A	< DL	< DL	< DL	< DL
α-Zearalenol*	---	---	---	---

* Not analyzed, only tested for cell proliferation.

Table 3. Occurrence and levels of chemicals in Missouri sewage treatment plants on August 8-9 2001. Concentrations were expressed as ng/L. DL = detection limit.

Chemical	St. Louis	Columbia	Independence	Kansas City
17β-Estradiol	< DL	< DL	< DL	< DL
Estrone	< DL	< DL	< DL	< DL
Estriol	< DL	< DL	< DL	< DL
17α-Ethinylestradiol	< DL	< DL	< DL	< DL
β-Estradiol Benzoate	< DL	< DL	< DL	< DL
Diethylstilbestrol	< DL	< DL	< DL	< DL
Permethrin	< DL	< DL	< DL	< DL
Bioallethrin	< DL	< DL	< DL	< DL
Fenvalerate*	---	---	---	---
Atrazine	4.3	< DL	1.7	4.2
Simazine	< DL	< DL	2.3	< DL
4-Octylphenol	<DL	4.8	<DL	2.0
Nonylphenols	6.5	139.5	2.9	43.6
4-tert-Octylphenol	< DL	3.2	<DL	< DL
Dibutyl phthalate	7.5	35.2	3.9	7.0
Butyl benzyl phthalate	3.0	2.0	1.9	7.8
Bisphenol A	< DL	68.5	< DL	< DL
α-Zearalenol*	---	---	---	---

* Not analyzed, only tested for cell proliferation.

Table 4. Occurrence and levels of chemicals in Missouri sewage treatment plants on October 18-19 2001. Concentrations were expressed as ng/L. DL = detection limit.

Chemical	St. Louis	Columbia	Independence	Kansas City
17β-Estradiol	< DL	< DL	< DL	< DL
Estrone	< DL	< DL	< DL	< DL
Estriol	< DL	< DL	< DL	< DL
17α-Ethinylestradiol	< DL	2.9	< DL	< DL
β-Estradiol Benzoate	< DL	< DL	< DL	< DL
Diethylstilbestrol	< DL	< DL	< DL	< DL
Permethrin	< DL	< DL	< DL	< DL
Bioallethrin	< DL	< DL	< DL	< DL
Fenvalerate*	---	---	---	---
Atrazine	1.9	0.6	< DL	< DL
Simazine	< DL	< DL	< DL	< DL
4-Octylphenol	0.8	6.5	4.6	1.3
Nonylphenols	13.3	226.1	101.9	30.1
4-tert-Octylphenol	1.1	3.9	3.0	1.4
Dibutyl phthalate	18.3	17.9	21.7	24.3
Butyl benzyl phthalate	5.1	3.3	4.8	5.4
Bisphenol A	< DL	18.4	< DL	< DL
α-Zearalenol*	---	---	---	---

* Not analyzed, only tested for cell proliferation.

Table 5. Estrogenic response of MCF7 cells to endogenous estrogens, synthetic estrogens, pesticides, herbicides, industrial chemicals, and one mycotoxin (α-zearalenol). The relative proliferative potency (RPP) or relative proliferative effect (RPE) values of each single chemical were calculated based on E2 in the corresponding solvent.

Chemical	EC50	RPP[1]	Ave. Max. Fold	RPE[1]
17β-Estradiol (in EtOH)	4.16 ± 1.84 pM	1.0	5.18 ± 1.03	1.0
17β-Estradiol (in DMSO)	2.16 ± 1.68 pM	1.0	3.42 ± 0.66	1.0
Estrone	214 ± 82 pM	0.02 ± 0.01	3.91 ± 1.07	0.76 ± 0.16
Estriol	53.5 ± 17.0 pM	0.09 ± 0.04	5.11 ± 0.70	1.02 ± 0.12
17α-Ethinylestradiol	2.86 ± 1.28 pM	0.94 ± 0.23	5.38 ± 0.56	1.08 ± 0.15
β-Estradiol Benzoate	10.4 ± 9.84 pM	0.71 ± 0.69	5.14 ± 0.88	0.93 ± 0.22
Diethylstilbestrol	23.7 ± 1.49 pM	0.14 ± 0.05	5.87 ± 1.40	1.01 ± 0.30
Permethrin	1.66 ± 1.46 μM	1.22E-5 ± 2.01E-5	2.97 ± 1.10	0.62 ± 0.06
Bioallethrin	N.E.[2]	N.E.	N.E.	N.E.
Fenvalerate	N.E.	N.E.	N.E.	N.E.
Atrazine	N.E.	N.E.	N.E.	N.E.
Simazine	N.E.	N.E.	N.E.	N.E.
4-Octylphenol	N.T.	N.T.	N.T.	N.T.
Nonylphenols	0.45 ± 0.61 μM	8.85E-5 ± 1.95E-4	3.94 ± 0.17	0.77 ± 0.99
4-tert-Octylphenol	0.31 ± 0.15 μM	2.02E-5 ± 9.60E-6	3.93 ± 0.66	0.81 ± 0.15
Dibutyl benzyl phthalate	N.E.	N.E.	N.E.	N.E.
Bisphenol A	0.28 ± 0.05 μM	2.8E-6 ± 2.4E-6	3.79 ± 0.03	0.94 ± 0.17
α-Zearalenol	59.8 ± 73.8 pM	0.23 ± 0.23	4.76 ± 0.57	1.03 ± 0.18

[1] The calculations for RPE and RPP were defined in the "*Quantitative Evaluation and Statistics*."
[2] N.E. denotes not estrogenic.

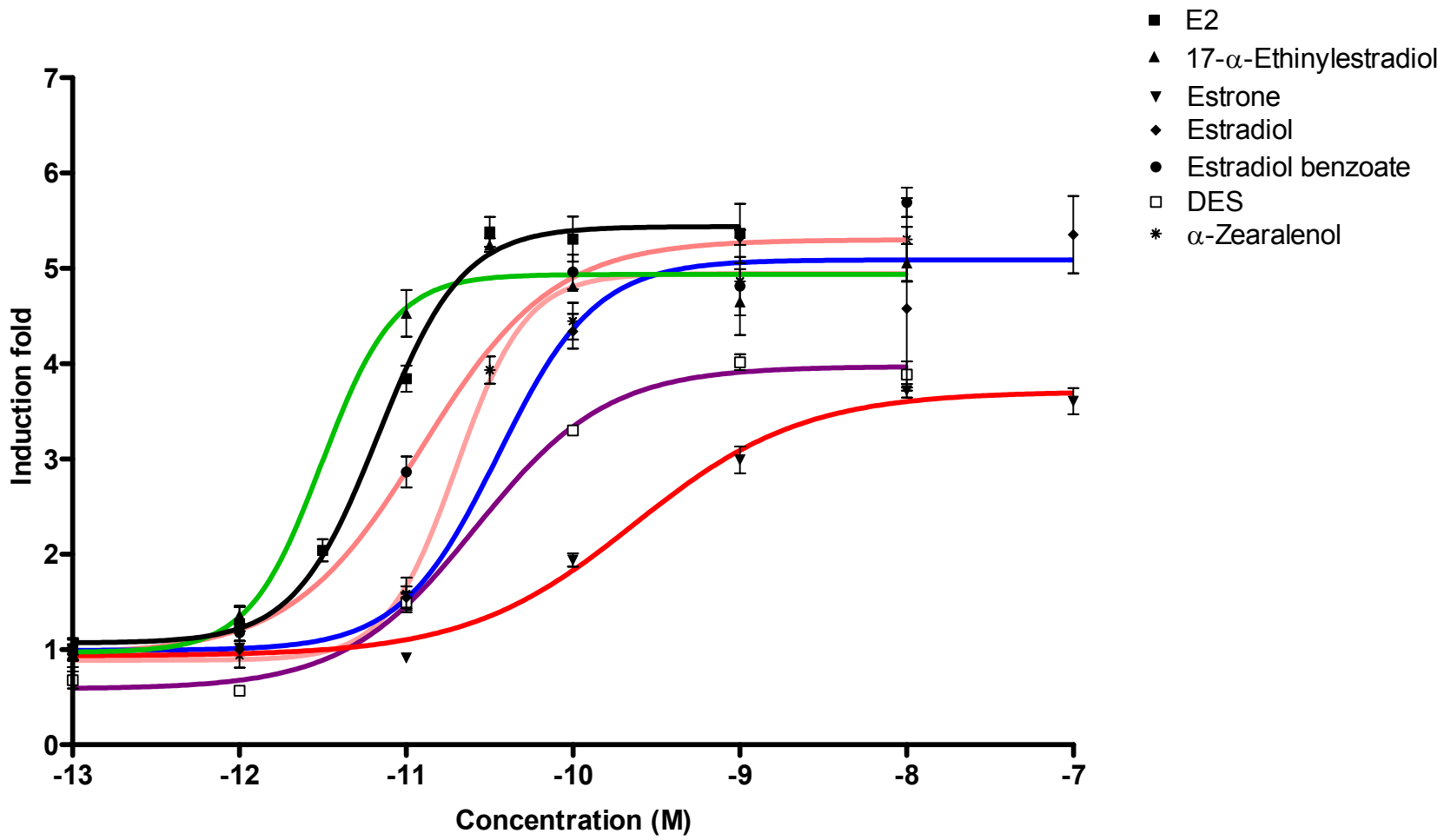

Figure 1. Representative concentration-response curves of the chemicals with strong estrogenicity on the proliferation of MCF7 cells. Cells were stained with sulforhodamine B, and the cell density was determined by a spectrophotometer.

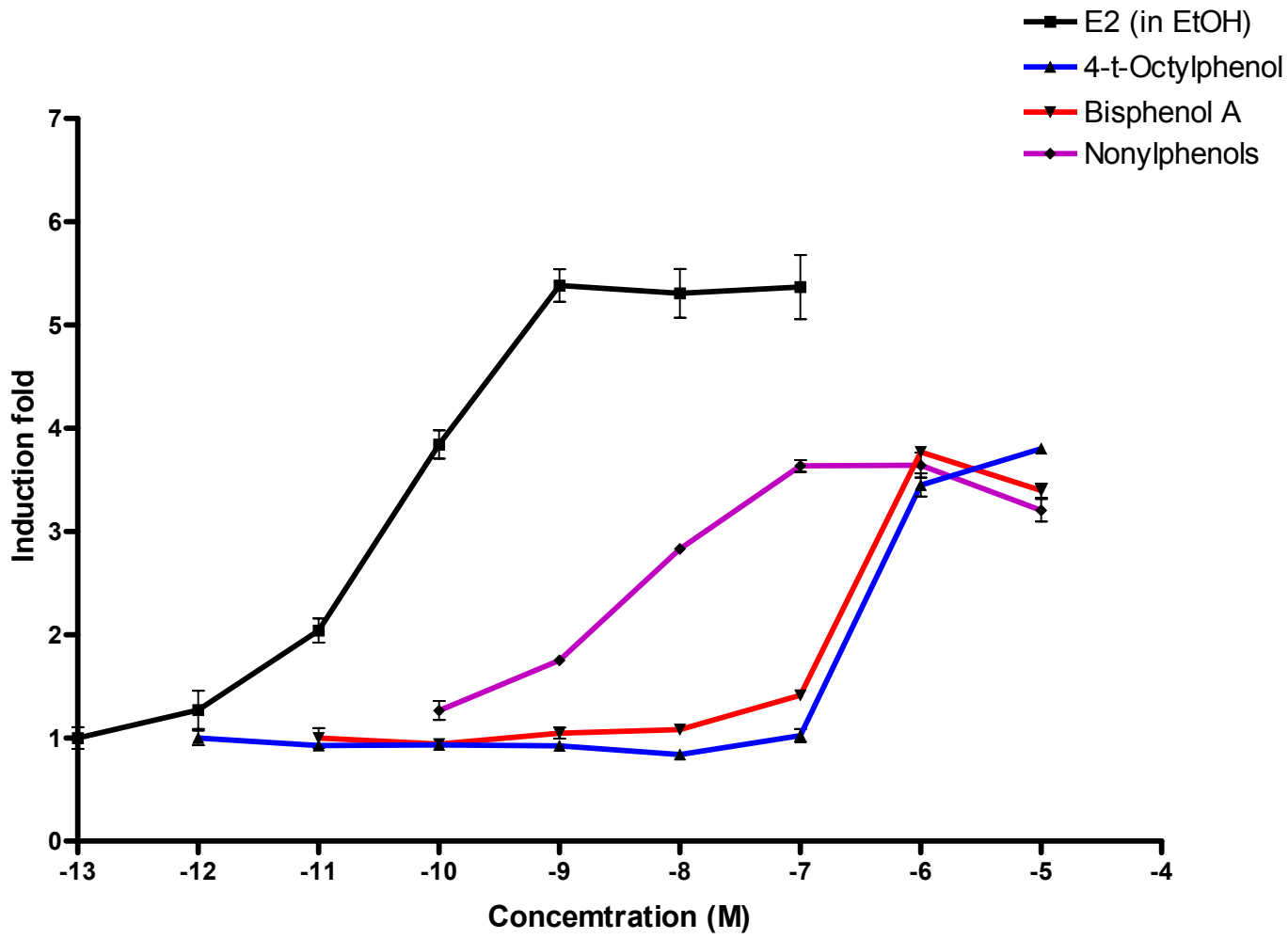

Figure 2. Representative concentration-response curves of the chemicals with weak estrogenicity on the proliferation of MCF7 cells.

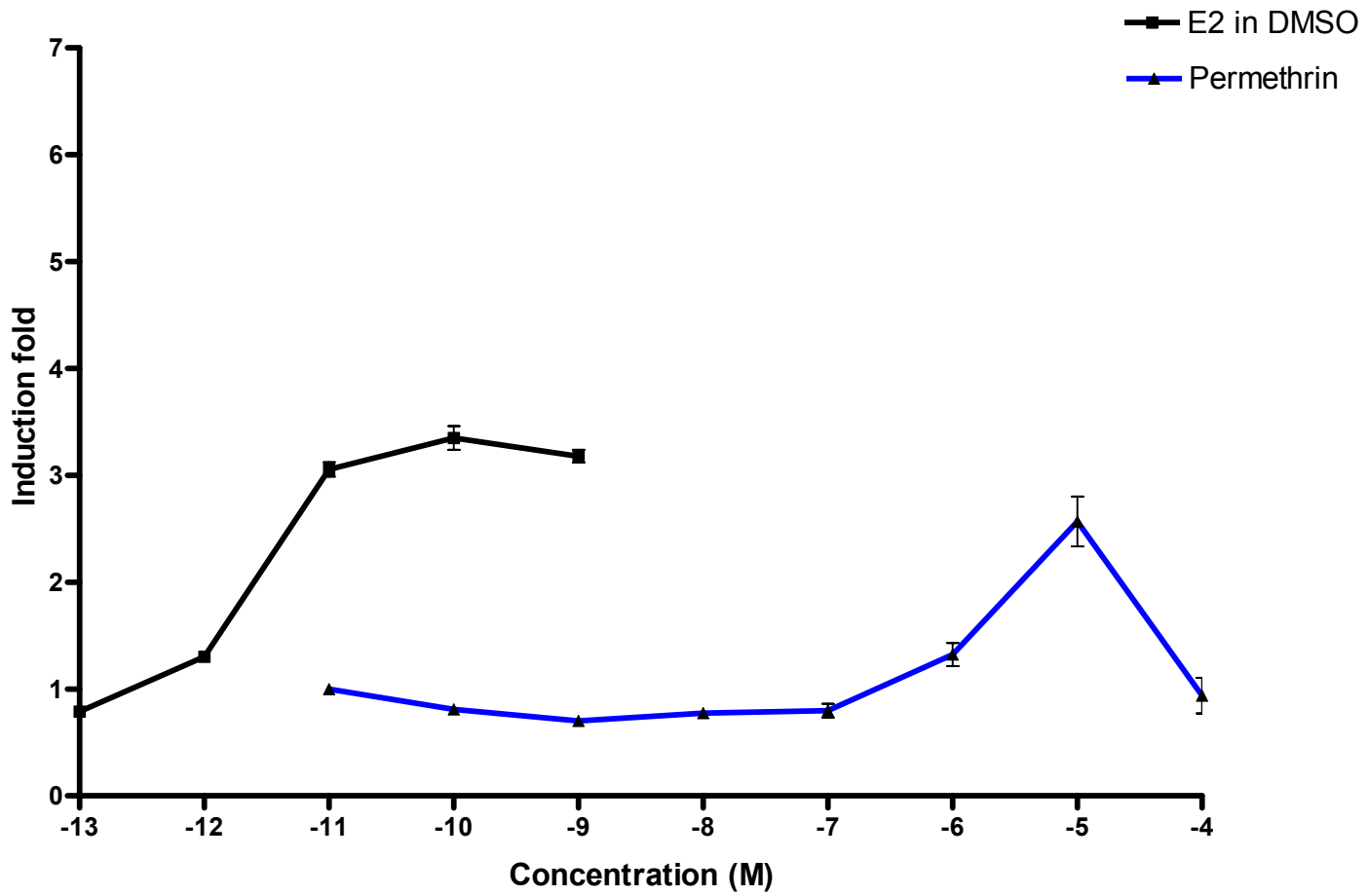

Figure 3. A representative concentration-response curve of permethrin, a weak estrogen, on the proliferation of MCF7 cells.

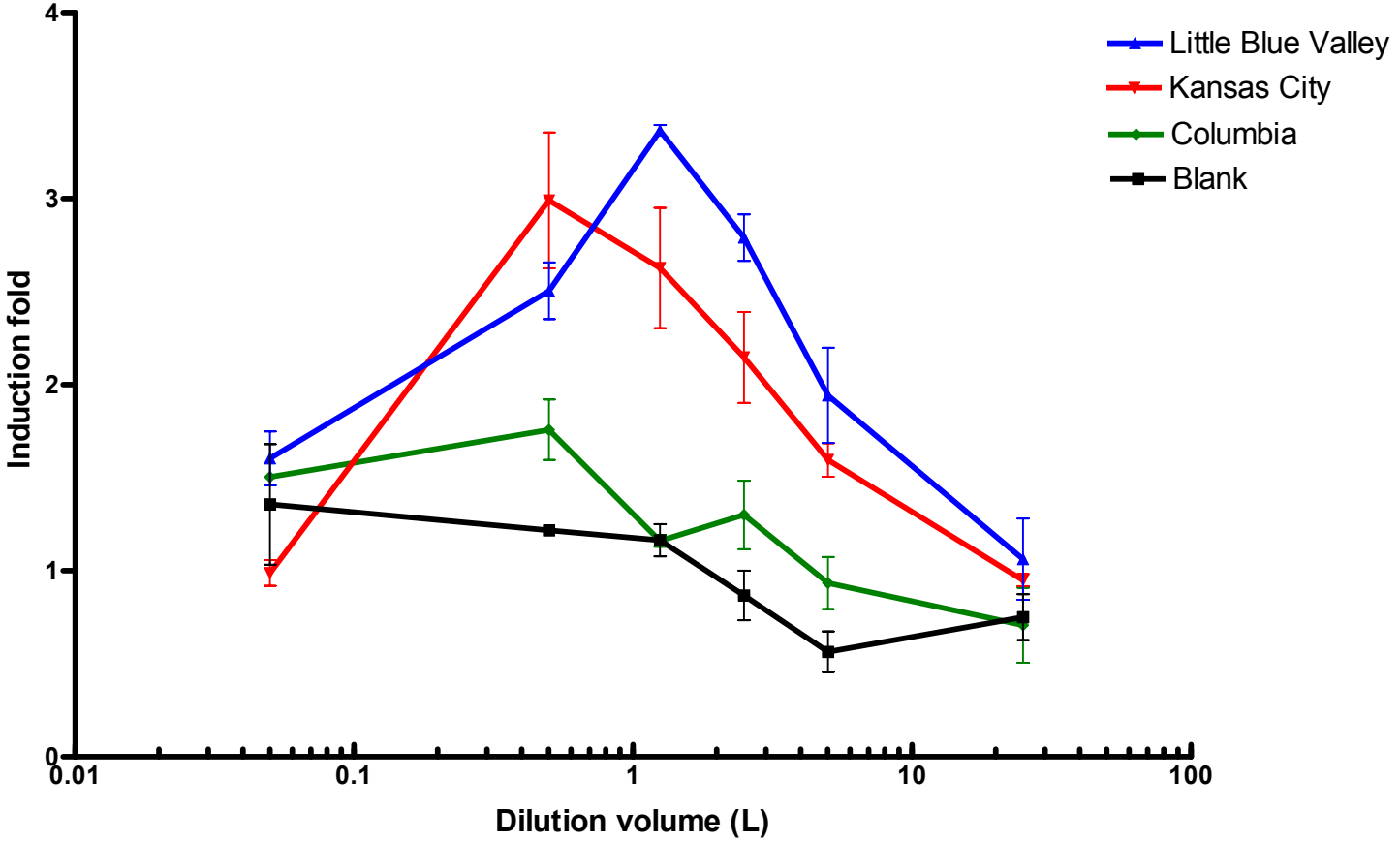

Figure 4. Representative concentration-response curves of the effluent extracts collected on Oct. 18 - 19, 2002. Inset is the E2 in DMSO as a standard curve. The sample from St Louis STP was lost in the preparation for tissue culture experiment. The plot of the dilution volumes was log transformed. Values represent means ± S.D. of triplicates in one single experiment.