 © United States Government 2012 Journal of Water and Health | 10.1 | 2012

Removal of estrogens and estrogenicity through drinking water treatment

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ABSTRACT

Estrogenic compounds have been shown to be present in surface waters, leading to concerns over their possible presence in finished drinking waters. In this work, two *in vitro* human cell line bioassays for estrogenicity were used to evaluate the removal of estrogens through conventional drinking water treatment using a natural water. Bench-scale studies utilizing chlorine, alum coagulation, ferric chloride coagulation, and powdered activated carbon (PAC) were conducted using Ohio River water spiked with three estrogens, 17β-estradiol, 17α-ethynylestradiol, and estriol. Treatment of the estrogens with chlorine, either alone or with coagulant, resulted in approximately 98% reductions in the concentrations of the parent estrogens, accompanied by formation of by-products. The MVLN reporter gene and MCF-7 cell proliferation assays were used to characterize the estrogenic activity of the water before and after treatment. The observed estrogenic activities of the chlorinated samples showed that estrogenicity of the water was reduced commensurate with removal of the parent estrogen. Therefore, the estrogen chlorination by-products did not contribute appreciably to the estrogenic activity of the water. Coagulation alone did not result in significant removals of the estrogens. However, addition of PAC, at a typical drinking water plant dose, resulted in removals ranging from approximately 20 to 80%.

Key words | chlorine, drinking water treatment, estrogenic activity, estrogens

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INTRODUCTION

Many of the chemicals identified as known or potential endocrine disrupting compounds (EDCs) may be present in surface waters used as drinking water sources due to their introduction from domestic and industrial sewage treatment systems and wet-weather runoff. Among the EDCs shown to be present in surface waters in the USA (Snyder *et al.* 1999; Kolpin *et al.* 2002; Bennotti *et al.* 2009), the naturally occurring and synthetic estrogens are of particular concern due to their documented effects in the environment (Jobling *et al.* 1998; Kidd *et al.* 2007), and in laboratory studies conducted at environmentally relevant concentrations (Biales *et al.* 2007; Lange *et al.* 2009). Additionally, reports in the popular print and broadcast media of possible risks to human health have been increasing. Although there has not yet been a final determination of risks posed by EDCs in finished drinking waters, it is prudent to explore if commonly employed drinking water treatment processes can remove EDCs.

Conventional treatment, used by the majority of surface water treatment plants in the USA, usually includes rapid mixing, coagulation, flocculation, sedimentation, filtration and disinfection. The formation of coagulant floc and its removal can remove certain organic molecules that associate with the floc material either through precipitation or adsorption mechanisms. Oxidants such as chlorine and adsorbents such as powdered activated carbon (PAC) are

doi: 10.2166/wh.2011.135

commonly added during the rapid mixing step to enhance the removal of organic contaminants. In the USA, pre-chlorination (chlorine prior to sedimentation) is used by 34% of all surface water treatment systems according to the Community Water System Survey conducted in 2000 (US EPA 2002). The percentages are higher for larger systems, with 45% of those serving populations over 500,000 and 52% of systems serving 100,001–500,000 using pre-chlorination. The same survey reports the use of PAC by 20% of surface water treatment systems serving populations over 500,000. Chlorination and PAC treatments have been shown to chemically change or remove organic contaminants.

Previous laboratory studies have evaluated the removal of EDCs, including the natural and synthetic estrogens, by various individual drinking water treatment processes. These reports have included the use of coagulants (Westerhoff *et al.* 2005; Chen *et al.* 2007), PAC (Yoon *et al.* 2003; Westerhoff *et al.* 2005) and chlorine (Westerhoff *et al.* 2005) in natural and model waters. These studies generally found that estrogens were removed by PAC and chlorine, but not coagulants. These studies did not identify by-products or utilize bioassays to determine estrogenicity removal.

The production of by-products and the related change in estrogenicity, as determined by bioassays, from the reaction of chlorine with estrogens has also been investigated (Hu *et al.* 2003; Lee *et al.* 2004; Moriyama *et al.* 2004; Liu *et al.* 2005; Lee *et al.* 2008). All of these studies were conducted in laboratory grade water to facilitate the evaluation of the chemical nature of the chlorination by-products. Depending upon the reaction conditions, some of the by-products retained estrogenic activity as determined by these assays. Only Liu *et al.* (2005) used a human cell line bioassay (MCF-7). The others used either a yeast or estrogen binding assay. Therefore, to our knowledge, no one has completed a drinking water treatment study for the removal of estrogens and estrogenicity using a natural water and human cell line bioassays. Using a natural water is important because it takes into account the reaction of free chlorine with the natural organic matter. It is possible that the natural organic matter has higher rates of reaction with chlorine as compared to the estrogens, thereby limiting the reaction of estrogens with chlorine. The use of human cell line bioassays is important because the transport of compounds into mammalian cells differs from that in yeast cells (US EPA 1998). Also, these assays, in contrast to yeast and binding assays, can distinguish between estrogen agonists and antagonists (US EPA 1998). This is necessary because the chlorination of a parent estrogen could produce both agonist and antagonist by-products. A statistical analysis of the correlations between the estrogenic activities, as determined by the bioassays, and the parent estrogen concentrations, as determined analytically, allowed for the determination of any increase or decrease in estrogenic activity associated with the presence of estrogen chlorination by-products. The use of a statistical analysis in the interpretation of the data is needed due to the inherent variability in the bioassay data.

The removal of organic contaminants through the coagulation process (rapid mixing through sedimentation) can be well simulated by jar testing. In the present study, jar tests were used to evaluate the ability of the coagulation process, alone and in combination with chlorine or PAC, to remove three estrogens; 17β-estradiol (E2), 17α-ethynylestradiol (EE2), and estriol (E3) from Ohio River water. Treatment efficacy was evaluated analytically by comparing the estrogen concentrations before and after treatment. Samples treated with coagulant and chlorine were also evaluated for estrogenic activity that may be associated with the production of chlorination by-products using two *in vitro* human cell line bioassays, the MVLN reporter gene assay (Pons *et al.* 1990) and the MCF-7 cell proliferation assay (Wiese *et al.* 1992; Soto *et al.* 1995). These assays both use an estrogen receptor-positive human breast cancer cell line and are capable of distinguishing estrogen agonists and antagonists. The bioassay data was used in conjunction with the analytical data to determine if the levels of estrogenic activity observed in the chlorinated samples were statistically different than the levels of estrogenic activity that would be predicted based on the concentrations of the residual parent estrogen. An increase in the observed level of estrogenic activity relative to that predicted based on the residual parent estrogen concentration would suggest the formation of estrogenic chlorination by-products. A decrease in the observed level of estrogenic activity relative to that predicted could indicate the formation of by-products that are anti-estrogenic or interfere with the ability to respond to estrogens.

METHODS

Water quality analyses

Untreated Ohio River water was obtained from the Richard Miller Drinking Water Treatment Plant, Cincinnati, OH (www.cincinnati-oh.gov/water/pages/-3301-/). Batches of raw water were analyzed for turbidity, total particles, total organic carbon (TOC), alkalinity, and pH. Turbidity was analyzed by the nephelometric method (Standard Method 2130 B) and total particles were analyzed by light obscuration (Standard Method 2560 C) *Standard Methods* APHA/AWWA/WEF (1995). The persulfate-ultraviolet oxidation method (Standard Method 5310 C) was used for the analysis of unfiltered samples for TOC. Alkalinity was analyzed by titration with sulfuric acid (Standard Method 2320 B). Sample pH was determined by the electrometric method (Standard Method 4500-H^+ B). Settled water was characterized by turbidity, total particles, and residual free chlorine (Standard Method 4500-Cl F), if applicable.

Jar tests

The treatments evaluated in each jar test, including the doses of coagulant, PAC and chlorine used, and the raw and settled water qualities are shown in Tables 1–3. The coagulants evaluated were aluminum sulfate (Alum, $Al_2(SO_4)_3 \cdot nH_2O$ $n =$ approx 12–14) and ferric chloride ($FeCl_3 \cdot 6H_2O$), both technical grade, from Fisher Scientific (Pittsburgh, PA). Coagulant doses were selected based on turbidity removal, which was evaluated for each batch of Ohio River water prior to use in jar tests. The coagulant dose was the lowest dose that achieved a target settled turbidity of 1–3 nephelometric turbidity units (NTU) under the mixing and settling conditions described below.

PAC, Hydrodarco B (Norit Americas Inc., Marshall, TX) was added as a slurry (20 mg/mL) during the rapid mixing step in selected jar tests. The final PAC concentration of 10 mg/L in the jar tests is within the range frequently used by full scale treatment plants (Yoon *et al.* 2003; Synder *et al.* 2007).

Chlorine doses of 2–3 mg/L were used in selected jar tests as shown in Table 3. Chlorine stock solutions, prepared from 4–6% (wt/v) reagent grade sodium hypochlorite (Fisher Scientific), were added to the treatment jars during the rapid mixing step. Stock solution concentrations were verified by iodometric assay (Standard Method 4500-Cl B) prior to use *Standard Methods* (APHA/AWWA/WEF 1995). Chlorine was dosed to achieve a target residual of 1 mg/L at the end of the settling period.

Estrogens were obtained from Steraloids (Newport, RI). Two estrogen fortification levels were used in the jar tests. Jar tests E2-A, E2-B, E2-C E2-D, EE2-A, EE2-B, EE2-C, and E3-A (Tables 1–3) were conducted using a nominal estrogen concentration of 0.5 μg/L. The estrogen concentration was increased to 1 μg/L in all subsequent jar tests, to ensure that the post-chlorination concentrations of the parent estrogen would be detectable by the analytical method used in this study.

Table 1 | Water quality, treatment parameters, and percent removals for jar tests using coagulant only

	Raw water quality						Settled water quality		
Jar test	Turbidity (NTU)	Particles × 10^4 (#/ml)	TOC (mg/L)	Alkalinity (mg/L as $CaCO_3$)	pH	Coagulant dose (mg/L)	Turbidity (NTU) Mean (RPD)[a]	Particles × 10^4 (#/ml) Mean (RPD)	% Estrogen removal based on LC/MS analysis (uncertainty)
EE2-A[b]	80.4	No data	No data	No data	No data	Alum 30	No data	No data	3.0 (6.7)
						Ferric[c] 40	No data	No data	0.5 (5.0)
E2-A	46.3	30.5	1.92	52	7.6	Alum 20	2.18 (11%)	1.40 (7.9%)	− 3 (10)
						Ferric 30	1.42 (11%)	0.905 (16%)	− 5.6 (8.1)
E3-A	39.2	23.0	1.92	52	7.6	Alum 20	1.70 (15%)	0.950 (5.4%)	2.7 (6.2)
						Ferric 30	1.18 (23%)	0.731 (17%)	3.8 (8.9)

[a]Relative percent difference in duplicate jars.
[b]No particle count, TOC, alkalinity, or pH data was collected for jar test EE2-A.
[c]Ferric chloride.

Table 2 | Water quality, treatment parameters, and percent removals for jar tests using coagulant with PAC 10 mg/L

	Raw water quality						Settled water quality		
Jar test	Turbidity (NTU)	Particles × 10^4 (#/ml)	TOC (mg/L)	Alkalinity (mg/L as $CaCO_3$)	pH	Coagulant dose (mg/L)	Turbidity (NTU) Mean (RPD)[a]	Particles × 10^4 (#/ml) Mean (RPD)	% Estrogen removal based on LC/MS analysis (uncertainty)
EE2-B	8.19	7.2	1.75	58	7.9	Alum 10	2.16 (11%)	2.2 (4%)	59 (6.7)
						Ferric[b] 10	2.81 (30%)	2.9 (28%)	59 (5.3)
E2-B	4.81	4.4	1.75	58	7.9	Alum 10	1.43 (39%)	1.1 (32%)	74 (11)
						Ferric 10	2.20 (11%)	2.0 (5%)	77 (9.9)
E2-C	37.1	45.4	2.38	72	8.1	Alum 20	1.40 (4%)	1.48 (6%)	48 (5.2)
						Ferric 20	2.10 (0%)	1.63 (0%)	29 (13)
E3-A	39.2	23.0	1.92	52	7.6	Alum 20	1.30 (1%)	0.888 (4.3%)	30 (5.2)
						Ferric 30	1.24 (27%)	0.818 (13%)	23 (4.7)

[a]Relative percent difference in duplicate jars.
[b]Ferric chloride.

Prior to beginning the jar tests, the estrogen was added in an acetone solution to the bottom of a glass 20 L carboy. The acetone was allowed to evaporate to avoid introduction of solvent into the water. Twenty liters of unfiltered raw water equilibrated to room temperature (20–23 °C) was added and the solution was mixed for 2 hours to attain a nominal concentration of 0.5 or 1 μg/L. Experiments were conducted at room temperature and ambient pH (7.6–8.2). Aliquots of 2 L of the fortified river water were transferred into glass beakers (jars) and placed on six position gang stirrers with stainless steel paddles (Phipps and Bird, Richmond, VA). All steel paddles, 2 L jars, and 20 L carboys were muffled at 400 °C for 1 hour prior to use in each jar test. Duplicate jars were used for each treatment combination. One set of duplicate jars in each jar test served as the controls and received no treatment. An additional control sample was taken directly from the carboy after the 2 hours of mixing.

Coagulant was added during rapid mixing (100 RPM for 90 s). PAC or chlorine, if used, was also added during rapid mixing. The rapid mix was followed by three sequential 10 min flocculation steps at 30, 20, and 10 RPM. The water was allowed to settle for 60 min after the last flocculation step. Samples for estrogen analysis (500 mL) were decanted from the control jars at the beginning of the jar test and at the conclusion of the settling period. All other samples were collected after settling. For samples containing chlorine, quenching agent was added, either 4 mg sodium metabisulfite (Fisher Scientific, ACS Certified Grade) or 2 mg sodium ascorbate (Fluka Chemical, St. Louis, MO) to each 500 mL sample bottle prior to sample addition. Settled turbidity, total particle counts, and residual free chlorine, if applicable, were determined for each treatment jar. Two samples were taken for instrumental analysis for estrogens from each treated jar.

Extraction and instrumental analysis

Aqueous samples were concentrated by solid phase extraction using C18 50 mm disks (J.T. Baker, Phillipsburg, NJ) that had been washed and conditioned with methanol. Disks were eluted with methanol. For selected jar tests, 20% of the extract was removed for bioassay, concentrated to dryness, and resuspended in ethanol (Aaper, Shelbyville, KY) as described below. The remaining extract was concentrated under nitrogen at 35 °C to a final volume of 1 mL, or 0.8 mL if an aliquot was removed for bioassay. Prior to analysis, internal standards consisting of deuterated analogues (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) of each estrogen were added to the extracts. Separation was performed using an XTerra C18 MS column (Waters, Milford MA) at 40 °C using a 0.25 mL/min flow rate and a water/methanol gradient with a constant concentration of 0.08% ammonium hydroxide, with an initial methanol concentration of 40%, increasing to 52% at 5 min. The column was washed with 75% methanol following elution of the target estrogen. Detection was performed by electrospray LC/MS using a Waters Micromass ZQ in the selected

Table 3 | Water quality, treatment parameters, and percent removals for jar tests using coagulant with chlorine or chlorine alone

	Raw water quality							Settled water quality			
Jar test	Turbidity (NTU)	Particles × 10^4 (#/ml)	TOC (mg/L)	Alkalinity (mg/L as $CaCO_3$)	pH	Coagulant dose (mg/L)	Cl_2 dose (mg/L)	Turbidity (NTU) Mean (RPD)[a]	Particles × 10^4 (#/ml) Mean (RPD)	Free Cl_2 Residual Mean (mg/L) (RPD)	% Estrogen removal based on LC/MS analysis (uncertainty)
EE2-C	9.32	20.3	2.68	74	8.2	Alum 30	3	0.76 (29%)	0.835 (11%)	No data	98 (6.4)
						Ferric[b] 30	3	1.68 (34%)	2.02 (52%)	No data	98 (6.4)
						None	3	7.85 (1.3%)	19.0 (5%)	No data	98 (6.4)
EE2-D	2.94	3.77	2.68	74	8.2	Alum 30	3	0.40 (23%)	0.297 (25%)	0.9 (0%)	98 (7.0)
						Ferric 30	3	0.95 (2%)	0.917 (6%)	0.6 (5%)	98 (7)
						None	3	2.29 (2%)	3.07 (0.2%)	0.7 (4%)	98 (7.0)
EE2-E	77.2	85.9	2.29	52	8.0	Alum 40	3	2.66 (20%)	2.16 (36%)	1.5 (0%)	98 (5.6)
						Ferric 40	3	2.42 (5.4%)	1.56 (30%)	1.4 (0%)	98 (5.6)
EE2-F	0.518	1.16	No data	73	7.9	Alum 5	2.25	0.84 (3%)	1.52 (3%)	0.8 (6%)	99 (18)
						Ferric 5	2.25	1.34 (5%)	1.87 (15%)	0.8 (1%)	99 (18)
E2-D	3.19	5.65	2.68	74	8.2	Alum 30	3	0.85 (31%)	0.474 (13%)	1.1 (18%)	99 (5.3)
						Ferric 30	3	0.99 (4%)	0.914 (12%)	0.8 (8%)	99 (5.3)
						None	3	2.96 (0.3%)	5.44 (3.1%)	1.0 (11%)	99 (5.3)
E2-E	37.5	53.3	1.95	51	7.6	Alum 20	2.5	3.01 (40%)	3.98 (37%)	1.0 (0.0%)	99 (9.8)
						Ferric 20	2.5	3.92 (2%)	4.25 (0.5%)	1.0 (4%)	98 (9.7)
E2-F	2.41	5.59	2.06	60	8.0	Alum 15	2.5	0.49 (0.4%)	0.505 (5.5%)	0.9 (5%)	99 (9.2)
						Ferric 15	2.5	1.22 (11%)	0.972 (14%)	0.9 (0.0%)	99 (9.2)
E3-B	1.14	1.65	2.71	80	7.8	Alum 20	3	0.92 (4%)	0.727 (18%)	1.0 (0.0%)	99 (28)
						Ferric 20	3	1.44 (16%)	1.26 (10%)	0.9 (12%)	99 (28)
						None	3	2.09 (3%)	2.48 (1%)	1.0 (30%)	99 (28)
E3-C	1.66	4.89	2.21	67	7.9	Alum 10	2.3	0.86 (3%)	0.862 (1%)	0.8 (10%)	99 (7)
						Ferric 10	2.3	1.67 (28%)	1.38 (30%)	0.8 (2%)	99 (7)
E3-D	1.93	1.98	No data	78	7.8	Alum 15	3	0.57 (18%)	0.494 (23%)	0.8 (6%)	98 (5)
						Ferric 15	3	1.22 (7%)	1.01 (11%)	0.8 (6%)	98 (5)

[a]Relative percent difference in duplicate jars.
[b]Ferric chloride.

ion mode. Quality assurance criteria included the use of an extraction surrogate, bisphenol A-d16, to evaluate recovery. Bisphenol A- d16 was fortified at 250 ng/500 mL in all controls and test samples. Surrogate recoveries of 70–130% were achieved for all data. Bioassay of the laboratory blanks showed no detectable estrogenic response attributable to bisphenol A-d16 at this concentration. Additional quality assurance controls included duplicate samples from each jar, laboratory blanks, and laboratory fortified river water and reagent water. The laboratory fortified controls were spiked with the test estrogen at a level similar to that in the treatment jars. Standard deviations for percent removals shown in Tables 1–3 were calculated to account for uncertainty propagation (Bevington & Robinson 1992).

Characterization of chlorination products

Preliminary experiments were conducted to characterize estrogen chlorination products in organic free reagent water using both the Waters/Micromass single quadrupole LC/MS and a Bruker Daltonics UltroTOFQ quadrupole/time of flight (Q-TOF) (Billerica, MA). In the absence of matrix interferences, the masses of the chlorination products were readily identified. This information was then used to screen for the presence of the identified estrogen chlorination products in the treated jar test samples.

Determination of the accurate mass of the chlorination product of E2 was performed using the Q-TOF with negative electrospray ionization and the quadrupole operating in the pass through mode. The Q-TOF parameters were optimized to achieve a mass resolution in excess of 20,000 while scanning over a mass range between 120 and 645. External mass calibration was performed using Agilent MMi-Low Concentration Tuning Mix (Santa Clara, CA).

An evaluation of the stability of the EE2-chlorination by-products in the presence of excess dechlorination agents was conducted because halogenated organics are known to react with sulfite (Bauman & Stenstrom 1989; Croue & Reckhow 1989) and thiosulfate (Gan *et al.* 2002). Thus, the dechlorination agent, by creating reaction products that would not be formed during water treatment, could alter the biological activity of chlorinated water samples. The biological activity of chlorinated mixtures, as measured in mutagenicity assays, has been shown to decrease following the addition of dechlorination agents (Cheh *et al.* 1980; Wilcox & Denny 1985). To evaluate the potential for reaction with dechlorination agents, solutions of EE2 (100 μg/L) were reacted with chlorine in reagent water for 90 min followed by the addition of excess metabisulfite, thiosulfate, or sodium ascorbate. These test solutions were extracted and analyzed by full scan using the single quadrupole LC/MS. The addition of sodium metabisulfite to the solution containing the chlorination products of EE2 yielded polar products, which by mass and isotope abundance ratios were consistent with the addition of sulfite ion to chlorinated EE2. Similarly, excess thiosulfate yielded polar products that had masses and isotope abundance ratios consistent with the formation of Bunte salts, which are formed from the reaction of thiosulfate with organohalides. The addition of excess sodium ascorbate to the chlorination products of EE2 did not result in the detection of new products. Therefore, sodium ascorbate appeared to be the best available approach to stopping the chlorination reaction while maintaining the chemical composition of the chlorination products. All jar tests in which the samples were submitted for bioassay used sodium ascorbate for dechlorination.

Bioassay methods

The MVLN reporter gene assay and the MCF-7 cell proliferation assay were used to evaluate the estrogenic activity of the mixtures of products formed following chlorination for jar tests E2-E, E2-F, EE2-E, and E3-D. Samples from jar tests EE2-F and E3-C were assayed using the MVLN bioassay only. Both chlorinated test samples and non-chlorinated controls were submitted for bioassay. One sample from each of the jars treated with coagulant and chlorine were submitted for bioassay. Control samples included extracts from the control jars at initial and final time points and extracts from reagent water fortified at 20 ng/L, consistent with the expected concentrations of E2, EE2, or E3 following chlorination.

Aliquots of the extracts of controls and treated samples were prepared for bioassay by dissolving the residue in ethanol. The final concentration of the parent estrogen in the wells of the bioassay plate was adjusted by dilution of

the sample and produced a response within a factor of six of the EC50 for all samples and controls except blanks. For jar tests using E2 and EE2, the extraction and bioassay procedures resulted in final concentrations in the wells such that the jar test controls were diluted 200–400-fold relative to the aqueous, and all other samples were diluted fourfold. Due to the lower estrogenic activity of E3, in samples for jar tests E3-C and E3-D the final concentrations in the wells represented a 40–80 fold dilution for jar test controls and a 1–2-fold concentration for all other samples. Within a jar test, dilution/concentration factors for controls and test samples were constant.

The culture and assay conditions for the cell lines, MVLN and MCF-7 subclone E3, have been previously described (Wiese *et al.* 1992; Rubin *et al.* 2002). Briefly, cultures were maintained in phenol red free Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and passed weekly. Prior to use in an assay, the MCF-7 and MVLN cells were withdrawn from media containing estrogen by passage into media containing dextran-coated charcoal stripped FBS (DCC media) for 5 or 6 days, respectively. These cells were washed three times daily with Dulbecco's phosphate buffered saline (DPBS) (Gibco) and the media was replaced with fresh DCC media for the first 4 days after cell attachment. Both assays were performed in Costar 96 well plates (Corning, Lowell, MA) seeded with withdrawn cells. The positive controls included E2 at 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M and the same doses of the estrogen evaluated in the jar test, if other than E2, that is either EE2 or E3. The negative controls included media and 0.1% ethanol blanks, and the anti-estrogen controls 10^{-7} M ICI-182,780 (Tocris Bioscience, Ellisville, MO) and E2 at 10^{-9} M in the presence of 10^{-7} M ICI-182,780.

In both the MVLN and the MCF-7 cell proliferation assays, each test sample or control was diluted into media to create a final concentration of 0.1% ethanol (v/v). A 100 μL aliquot of this dilution in media was transferred to each of four adjacent seeded wells, to form a set of quadruplicate treated wells. To reduce possible bias due to error in dilution of sample into media, each test sample was independently diluted into media between one and four times for each weekly experiment (cell passage); with each dilution tested in four replicate wells. Each test sample was evaluated in at least two cell passages (weeks of cell culture) on separate days for each cell line used.

For the MCF-7 proliferation assay, cells were plated on day 0 at a density of 1.8×10^5 cells/well, and dosed with the test extract dilutions on days 1 and 4. On day 7 the number of metabolically active cells was determined indirectly by the detection of the fluorescent product of Alamar Blue dye (Trek Diagnostic Systems, Cleveland, OH) using a Dynatech Fluorolite 1000 fluorescence reader (Burlington, MA). For the MVLN reporter gene assay, cells were plated on day 0 at 1.5×10^6 cells/well and formation of confluent monolayers was verified on day 1 prior to addition of test sample dilutions. Media was removed and cells were dosed a second time on day 2. On day 3 media was removed, cells were washed 2 times with DPBS, lysed, and luciferase activity was measured directly in the wells by automated addition of luciferin substrate (Promega Corp. Madison, WI) followed by measurement of light production on a Dynex MLX luminometer (Chantilly, VA). All data was collected electronically.

Statistical analysis of bioassay results

A standard curve based on a logistic dose-response was generated from positive controls representing five log 10 dose levels of the estrogen used in each jar test, using Prism 4 software (GraphPad, San Diego, CA). Equivalent estrogen concentration for each of the four replicate sample or control wells was based on interpolation from this standard curve. Final log10 equivalent estrogen concentration was taken as the median of these four replicate wells for the jar test samples and controls evaluated in the MVLN and MCF-7 cell proliferation assays.

Effects of coagulation and chlorination were modeled using a nested random effects analysis (SAS PROC MIXED, Version 9, SAS Institute, Inc., Cary, NC) based on three treatment types (controls, alum and chlorine, and ferric chloride and chlorine). Cell passage, jar number and independent dilutions of the sample extract were considered to constitute random effects. The analysis variable was the logarithm (base 10) of the ratio of estrogen equivalent activity (ng/L) measured in the bioassays, as determined above, to the parent estrogen concentration

(ng/L) determined by LC/MS. Separate analyses were performed for the two bioassays. Within each assay, analysis results are reported separately for individual jar tests. Differences in the log activity-concentration ratios due to each of the individual treatment conditions (alum or ferric chloride coagulant plus chlorine), the combined treatments (coagulant plus chlorine) and the control samples were evaluated at $\alpha = 0.05$.

In four cases for the MCF-7 assay, three or all four of the wells from the same dilution of a sample were out of the range of the standard curve. These cases were suspected of being in error due to the fact that the other three dilutions made from the same sample were well within the range of the standard curve. Studentized residuals for the median log concentration from each of the usually three or four dilutions for each sample were calculated based on the average log concentration for that passage and the pooled variance among all passages for the MCF-7 assay. These were compared to the Bonferroni-adjusted critical *p*-value of 0.00035, for an outlier test for 144 observations at the 0.05 critical level. *p*-values for the four suspect observations ranged from 0.00016 to 0.000003, well below the critical level, with the next lowest *p*-value among all other observations being 0.025. Based on this, the four observations were not used in the analysis. Their inclusion in the analysis would not materially affect any conclusions, but would influence the precision estimates.

RESULTS AND DISCUSSION

Estrogen removal by biodegradation

Biodegradation of E2 in river waters has been reported (Jürgens *et al.* 2002). The transformation of E2 to estrone (E1) was reported to have half-lives of 0.2 to 9 days, with further degradation of E1 occurring at a similar rate. In the present study, biodegradation was evaluated by comparing the concentrations of the estrogens in the control jars (no treatment) at the beginning of the jar test and at the conclusion of the settling period (90 min). No significant decreases in the concentrations of the estrogens in the controls were observed in any of the jar tests. Based on the data from the control jars, it is unlikely that biodegradation contributed substantially to the estrogen removals reported in the present study.

Estrogen removal by coagulation

Coagulation alone, with either alum or ferric chloride, did not result in significant removals of the estrogens (Table 1). Westerhoff *et al.* (2005) have reported similar results using spiked river water. In their study, alum coagulation resulted in removals of 0 and 2% for EE2 and E2, respectively. Results following treatment with ferric chloride were comparable.

Estrogen removal by coagulation with addition of PAC

The addition of Hydrodarco-B PAC during coagulation resulted in removals of the three estrogens ranging from 23 to 77%, as shown in Table 2. The large range of removals observed over the four jar tests can be attributed to differences in water qualities and estrogen hydrophobicity. The effect of water quality on the efficacy of PAC for the removal of E2 can be seen by comparison of jar test E2-B to E2-C. Jar test E2-B used water with a turbidity of 4.8 NTU and yielded 74 and 77% removals with alum and ferric chloride, respectively. Jar test E2-C used water with a turbidity of 37.1 NTU and yielded 48 and 29% removals with alum and ferric chloride. Using a water with a turbidity of 39 NTU, jar test E3-A resulted in estrogen removals of 30 and 23%, depending upon the coagulant. E3 is less hydrophobic than E2 or EE2. This may have contributed to the lower observed removals. The impact of water quality is consistent with previous reports in which the removal of small organic molecules using PAC was found to be dependent on the levels of natural organic matter (NOM) and turbidity (Bruce *et al.* 2002; Ho & Newcombe 2005). The decreased effectiveness of PAC in the presence of higher turbidities and NOM may be attributed to greater entrapment of the PAC particles within the floc structure and increased competition for PAC adsorption sites (Ho & Newcombe 2005). Results of the current study are also consistent with a report by Yoon *et al.* (2003) in which removals of 50 to 97% for E2 and EE2 were observed in two natural waters using Hydrodarco-B and doses of 5 and 15 mg/L.

Decrease in estrogen concentration by reaction with chlorine

The chlorination conditions used in the present study resulted in free chlorine residuals following settling of 0.6–1.5 mg/L, which is within the range typical of finished drinking waters in the USA (Summers *et al.* 1996). In all cases following treatment with coagulant and chlorine, or with chlorine alone, concentrations of the parent estrogens decreased by 98–99%, as shown in Table 3. The decrease in the parent estrogen was independent of the presence of coagulant. That along with the lack of removal with coagulation alone indicates that the observed reduction can be attributed to reaction with chlorine. Similar reductions in estrogen concentrations have been reported by Westerhoff *et al.* (2005) in natural waters in the presence of a chlorine residual.

The reduction in the concentrations of the parent estrogens following reaction with chlorine is associated with the production of chlorination by-products. The estrogenic activities of these by-products have previously been evaluated in relationship to the treatment conditions used (Lee B-C *et al.* 2004; Lee Y *et al.* 2008) and the chemical structure of the by-products formed (Hu *et al.* 2003; Liu *et al.* 2005). In the present study, the estrogenic activities of chlorination by-products produced under conditions simulating pre-chlorination of surface waters were evaluated using two human cell line bioassays. The chlorination products represent those present after a contact time of 90 min.

The extracts of the jar test samples treated with coagulant and chlorine were initially evaluated analytically for the presence of chlorination by-products based on information obtained from estrogen chlorination experiments conducted in organic free water and reports in the literature (Hu *et al.* 2003; Lee B-C *et al.* 2004; Moriyama *et al.* 2004; Liu *et al.* 2005; Lee Y *et al.* 2008). Chlorination by-products with masses and isotopic abundances consistent with the gain of two chlorines and one oxygen were detected in extracts of coagulated and chlorinated E2, EE2, and E3 jar test samples using the single quadrupole LC/MS. Verification that the by-product of E2 represented the gain of two chlorines and one oxygen was obtained using the Q-TOF. The monoisotopic mass of 355.0875 obtained for the negative ion of the by-product agreed with an elemental composition assignment of $C_{18}H_{21}Cl_2O_3$ within 2 ppm, and the isotopic abundance pattern was consistent with the addition of two chlorines. Thus, the chlorination by-product of E2 determined by LC/MS was confirmed by a second analytical method.

In order to characterize the estrogenic activity associated with the mixture of chlorination by-products present, including products that may not have been detected analytically, following treatment with coagulant and chlorine, the MVLN assay and the MCF-7 cell proliferation assay were used. The scatter plots in Figure 1 show the correlation between the estrogen equivalent activities as determined in the two bioassays and the concentrations of the parent estrogens, as determined by LC/MS, for samples from jar test EE2-E.

The jar test controls are extracts of Ohio River water fortified with EE2 at a nominal concentration of 1 μg/L. The lab fortified reagent water controls were fortified at a level of estrogen similar to the expected post-treatment estrogen levels (2% of the initial estrogen concentration) prior to extraction. Both controls contain only parent estrogen, since they were never treated with coagulant/chlorine, and were used to calculate a predicted estrogenic response based on the concentration of the parent estrogen. The predicted response line, shown in Figure 1, is the geometric mean of the ratios of the equivalent estrogenic activities to parent estrogen concentrations for the controls. The alum with chlorine and the ferric chloride with chlorine are extracts of the treated jar test samples, which contain residual parent estrogen and estrogen chlorination by-products.

As can be seen in the scatter plots, the observed reductions in estrogenic activity following treatment with coagulant plus chlorine are consistent with the 98% reductions in the analytically determined concentrations of EE2. The consistency between the observed levels of estrogenic activity for the jar test samples treated with coagulant and chlorine and the predicted response based on the controls suggests that the estrogen chlorination by-products present in the treated samples do not appreciably increase or decrease the overall level of estrogenic activity observed.

The results for the second jar test using EE2 showed a similar pattern of a decrease in estrogenic activity consistent with that expected based on the concentration of residual parent estrogen following coagulation/chlorination. Similar results were obtained for the jar tests conducted using E2 and E3. Lee *et al.* (2008) reported a similar pattern using

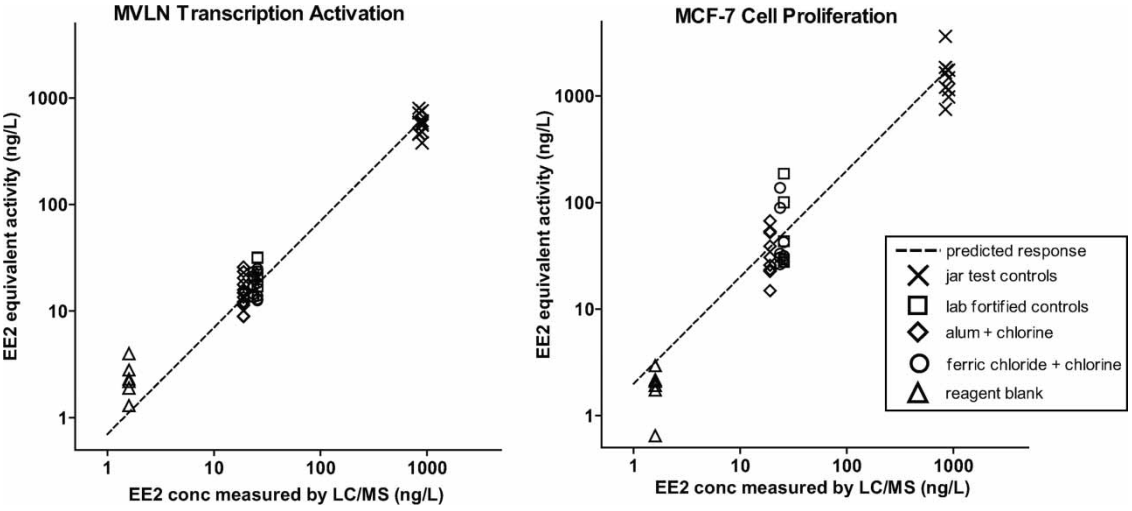

Figure 1 | Scatter plots showing the correlation between estrogen equivalent activity and analytical concentration for Jar Test EE2-E as measured by the MVLN and MCF-7 cell proliferation bioassays. Each data point represents the median of the equivalent estrogen activities of the quadruplicate wells multiplied by the appropriate dilution factors. The predicted response lines have a slope equal to the geometric mean of the ratios of the estrogen equivalent activities to the parent estrogen concentrations for the controls, which include the jar test controls and lab fortified controls. The laboratory fortified controls and ferric chloride with chlorine data overlap.

the yeast estrogen screen, in which there was a parallel decrease in relative EE2 concentration and estrogenic activity as a function of chlorine dose when the estrogen was reacted with chlorine in buffered water for 1–2 days.

It could be surmised that the reaction of chlorine with NOM did not produce a significant amount of estrogenic materials. However, the solid phase extraction method used was optimized to recover the parent estrogens. The disinfection by-products (DBPs) formed by the reaction of NOM with chlorine perhaps did not have similar chemical properties, which would allow them to first adsorb to the C18 disks, avoid elution from the disks with the methanol/water wash step, and avoid volatilization during the dry down procedure. Therefore, some DBPs that were estrogen agonists/antagonists may have been lost during the preparation of the samples. Another potential confounding factor is that the overall estrogenicity (relative potency × concentration) of the NOM-formed DBPs may have been orders of magnitude less than that of the parent estrogens, even after treatment where the parent estrogen was only a few percent of its original concentration. Both issues prevent a definitive conclusion from being made.

The scatter plots also show the greater variability in the MCF-7 cell proliferation assay compared to the MVLN assay under the conditions used in the current study. This difference in variability reflects differences in the complexity of the endpoints being evaluated and the potential for experimental variability between the two assays. Cell proliferation, a biologically relevant endpoint, requires a coordinated sequence of numerous events (Dees *et al.* 1997; Shappell 2006), compared to the less complex interactions required for reporter gene expression in the MVLN assay (Pons *et al.* 1990). In the cell proliferation assay, small differences in the initial number of cells seeded into the wells can lead to large differences in the final cell number after 7 days of incubation. In the MVLN assay, the cells are at confluence prior to treatment for 2 days. Thus, differences in the initial number of cells seeded do not have a substantial impact on the expression of the reporter gene during treatment.

To facilitate a statistical analysis of the contribution of the chlorination by-products to the estrogenic activity present following treatment, ratios of the estrogen equivalent activities to the parent estrogen concentrations were calculated for the treated jar test samples and compared to the predicted response based on the control ratios. Figure 2 shows the geometric means and associated 95% confidence intervals of the ratios for the controls, which are the predicted responses; the individual treatment conditions (alum or ferric chloride and chlorine); and the combined treatments (coagulant plus chlorine) for each jar test. These ratios differ from unity due to the uncertainty in the calculated dose–response curves and variability in the

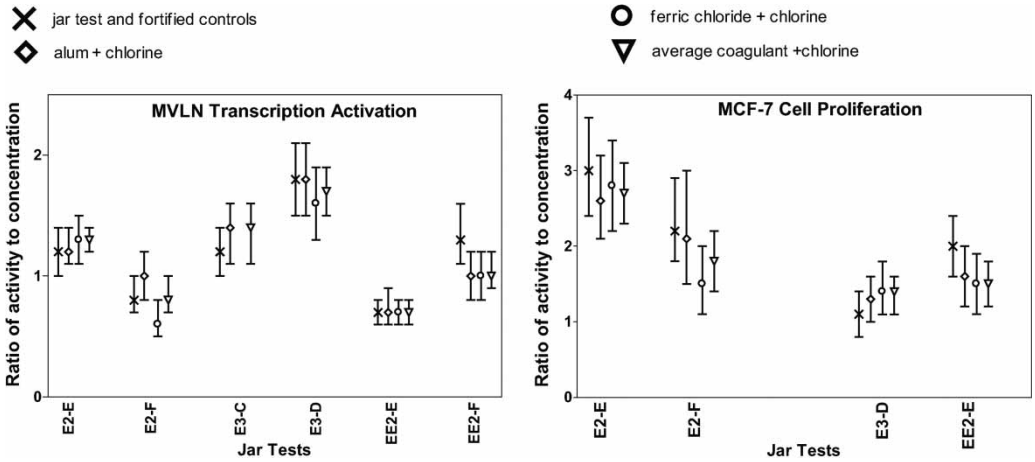

Figure 2 | Ratio of estrogen equivalent activity to analytically determined parent estrogen concentration in the MVLN and MCF-7 cell proliferation assays. Symbols represent the geometric means of the ratios and the bars represent the 95% confidence intervals.

bioassay data. The greater variability in the cell proliferation assay, compared to the MVLN assay, is evident from its wider confidence intervals in Figure 2. No significant differences in the ratios of the estrogen equivalent activities to the parent estrogen concentrations among samples treated with alum or ferric chloride and chlorine and the controls were seen for any of the individual jar tests ($p > 0.05$) in either the MVLN or MCF-7 cell proliferation assays. Additionally, no significant differences between all chlorinated samples, regardless of coagulant used, and the controls were seen. Together, these results indicate that the estrogen chlorination by-products do not increase or decrease the levels of estrogenic activity observed in the chlorinated water samples.

CONCLUSIONS

Treatment with coagulant alone, or in combination with PAC, was less effective than treatment with chlorine for the removal of three estrogens, E2, EE2, and E3, spiked into Ohio River water. Conditions simulating pre-chlorination of surface waters resulted in approximately 98% reductions in the concentrations of the parent estrogens, accompanied by the formation of by-products. The MVLN reporter gene assay and the MCF-7 cell proliferation assay were used to characterize the estrogenic activity of the water samples before and after chlorination. The observed estrogenic activities of the chlorinated samples showed that the estrogenic activity of the water was reduced commensurate with the removal of the parent estrogen. Therefore, the estrogen chlorination by-products did not contribute significantly to the estrogenic activity of the water. Given the parallel decreases in the concentrations of the steroid estrogens and the estrogenic activities observed in this study, the use of chlorine in drinking water treatment may serve to help manage possible estrogenic risks associated with the presence of steroid estrogens.

ACKNOWLEDGEMENT

The US Environmental Protection Agency, through its Office of Research and Development, funded and managed, or partially funded and collaborated in, the research described herein. It has been subjected to the Agency's administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the author(s) and do not necessarily reflect the views of the Agency, therefore, no official endorsement should be inferred. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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First received 2 March 2011; accepted in revised form 23 September 2011. Available online 16 October 2011