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SCREENING COMPLEX EFFLUENTS FOR ESTROGENIC ACTIVITY WITH THE T47D-KBLUC CELL BIOASSAY: ASSAY OPTIMIZATION AND COMPARISON WITH IN VIVO RESPONSES IN FISH

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Abstract—Wastewater treatment plant (WWTP) effluents can contain estrogenic chemicals, which potentially disrupt fish reproduction and development. The current study focused on the use of an estrogen-responsive in vitro cell bioassay (T47D-KBluc), to quantify total estrogenicity of WWTP effluents. We tested a novel sample preparation method for the T47D-KBluc assay, using powdered media prepared with direct effluent. Results of the T47D-KBluc assay were compared with the induction of estrogen receptor–regulated gene transcription in male fathead minnows (*Pimephales promelas*) exposed to the same effluents. Effluent samples for the paired studies were collected over the course of three months. According to the T47D-KBluc assay, the effluent estrogenicity ranged from 1.13 to 2.00 ng 17β-estradiol (E2) equivalents/L. Corresponding in vivo studies exposing male fathead minnows to 0, 10, 50, and 100% effluent dilutions demonstrated that exposure to 100% effluent significantly increased hepatic vitellogenin (VTG) and estrogen receptor α subunit transcripts relative to controls. The induction was also significant in males exposed to 250 ng E2/L or 100 ng E2/L. The in vitro and in vivo results support the conclusion that the effluent contains significant estrogenic activity, but there was a discrepancy between in vitro– and in vivo–based E2 equivalent estimates. Our results suggest that the direct effluent preparation method for the T47D-KBluc assay is a reasonable approach to estimate the estrogenicity of wastewater effluent. Environ. Toxicol. Chem. 2011;30:439–445.

Keywords—Estrogens Gene expression Complex mixtures Solid-phase extraction

INTRODUCTION

Estrogens are steroid hormones that play important roles in the sex determination, reproduction, and development of sexual characteristics and behaviors in vertebrates, including fish. However, exposure to exogenous steroidal estrogens and other estrogenic contaminants in the aquatic environment has the potential to disrupt fish development and reproduction [1–3]. Of particular concern is the ability of estrogens to feminize male fish through disruption of testis growth by development of oocytes and stimulation of vitellogenin production [4]. Effluents from wastewater treatment plants (WWTPs), in particular, have been shown to contain complex mixtures of endocrine-disrupting compounds [5–8], including steroidal estrogens such as 17β-estradiol (E2) and ethinylestradiol [6,9–11], as well as plant-derived phytoestrogens [12]. Feminization of male fish exposed to estrogenic effluents has been widely documented, with a number of studies showing sufficient levels of estrogenic compounds to produce adverse effects on reproduction [1,3,6,13,14].

After treatment, wastewater effluent is discharged into the environment, typically into lakes, rivers, or other bodies of water. Because estrogenic endocrine-disrupting compounds are capable of inducing physiological effects at low doses, one must evaluate the concentrations of estrogenic compounds in effluent to ascertain the possibility for potentially harmful effects on fish populations in the surrounding aquatic ecosystem where the effluent is discharged. One way to determine the estrogen content of wastewater effluent is to conduct chemical analyses. However, because of the complexity of most effluents, chemical analyses can be time consuming and difficult. Additionally, they generally require use of specific extraction and clean-up procedures, analytical instrumentation, and de facto a priori selection of target chemicals of interest within the sample. This is problematic, because many estrogenic chemicals may be unknown or are present in the environment in minute amounts that are difficult to detect with analytical techniques but that, individually or in combination, may cause effects. In vitro cell bioassays can be used to help address the shortcomings of targeted chemical analyses and provide an efficient, sensitive, and unsupervised method of measuring estrogenic activity within complex effluents that includes the potential interactive effects of multiple chemicals [15,16].

In the current study, we evaluated the T47D-KBluc cell assay, which is a relatively new and sensitive in vitro system [16], developed through stable transfection of the T47D human breast cancer cell line with a luciferase reporter gene under control of triplet estrogen response elements [17]. The T47D-KBluc assay was selected because it has been more reliable and sensitive to estrogens than several other in vitro screening methods [16,17], making it a useful tool for detecting estrogens in environmental samples. Our first aim was to establish an effective method of effluent sample preparation for the in vitro

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cell assay. We then evaluated the estrogenic activity of final wastewater effluents from a local WWTP receiving domestic and industrial pulp and paper mill inputs, over multiple time points, using paired in vitro and in vivo assays conducted with the same effluents. Because of particular concerns regarding the potential effects of estrogens on aquatic vertebrates, notably fish, we wanted to determine whether the in vitro results based on a human recombinant cell line would be predictive of estrogenic responses in fish.

Because gene expression changes can be used as a diagnostic tool to screen for the presence of estrogens in an unknown sample such as an effluent [18], the relative expression of two key indicator genes in mature male fathead minnow livers was determined. One significant indicator of estrogen exposure in fish involves induction of vitellogenin (VTG) in male fish [19–21]. In addition, estrogen receptor α (ESR1) messenger RNA levels are normally undetectable or quite low in male fathead minnow liver [22]. However, when male fish are exposed to estrogens such as E2 or 17α-ethinylestradiol (EE2), ESR1 transcript abundance in the liver increases [18]. Consequently, in our in vivo work, VTG and ESR1 expression were examined in the liver of male fathead minnows exposed to the test effluent, as well as the model estrogen 17β-estradiol (E2).

MATERIALS AND METHODS

Effluent sample collection

Treated wastewater effluent for the current study was collected at Western Lake Superior Sanitary District (WLSSD), a WWTP located in Duluth, Minnesota, USA, which treats mixed domestic and pulp and paper mill influent. This particular WWTP is a Class A facility that discharges to the St. Louis River, a major tributary to Lake Superior, as required by its National Pollutant Discharge Elimination System permit. It provides advanced secondary treatment of wastewater via the activated-sludge biological process, which is enhanced with pure oxygen (e.g., UNOX system). The calculated design wet-weather flow for the WWTP is 48.4 million gallons per day, and its average flow is 38.7 million gallons per day. The WWTP services a sewer-connected population of approximately 105,000 people. The sewer system to the WWTP conveys only wastewater, which is not a combined storm/sanitary system. The WWTP influent flow is approximately 50% municipal (residential and commercial) and 50% industrial. The major municipality (Duluth, MN, USA) is home to two large regional medical centers, and the major industry is pulp/paper production. One mill uses the Kraft process for pulp and produces high-quality coated paper, and a second mill produces lower-quality paper from recycled paper or pulp derived from a thermal/mechanical process.

On each sampling day, a 75-L grab sample of final treated effluent was collected by pump from the WLSSD pump house between 7:00 AM and 9:00 AM. Simultaneously, personnel at the U.S. Environmental Protection Agency Mid-Continent Ecology Division (MED) collected 95 L of onsite filtered and sterilized Lake Superior water (LSW) in Cubitainers for use in effluent dilutions and as controls. The LSW used at the Environmental Protection Agency MED facility is collected over 12 km away from the WLSSD effluent discharge site. Immediately after collection, the final effluent samples were transported back to MED in new Milli-Q (Millipore) water–rinsed Cubitainers (20 L), in which half of the effluent was mixed in a large glass tank for use in in vivo fish exposures, and 1 L was extracted (<2 h later), whereas the remaining effluent was stored at 4°C (<24 h) until needed for tank water renewal and the T47D-KBluc cell assay.

Effluent sample extraction

To prepare effluent extracts, 1 L effluent was filtered through a 1-μm filter and extracted at a flow rate of approximately 5 ml/min, using a 6-ml high-capacity C18 solid-phase extraction (SPE) disk (Baker Bond) that had been activated with methanol and Milli-Q water. The column was eluted with 4 × 1-ml aliquots of 100% methanol, which were combined and evaporated to 100 μl under a gentle stream of N_2 gas. The sample volume was brought to 200 μl with Milli-Q water. The final extract was diluted in 50% methanol to prepare extract concentrations ranging from 100% extract (5,000 ×) to 0.2% extract (10 ×). The extract was diluted by a factor of 1, 5, 10, 50, 100, or 500.

Direct effluent media preparation

As an alternative to SPE, we also examined the use of cell culture media directly prepared using whole effluent. We subsequently refer to this as direct effluent media. Dry media components (0.125 g glucose, 0.075 g sodium bicarbonate, and 0.52 g RPMI 1640 powder (Sigma) were measured and transferred to a 50-ml centrifuge tube to which 500 μl 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (1 M, GIBCO), 500 μl sodium pyruvate (100 mM, GIBCO), and 2,500 μl charcoal dextran–filtered fetal bovine serum (HyClone) were added. Media components were then dissolved in 50 ml effluent (100% effluent media), fresh LSW (LSW media; control), Cubitainer LSW (Cubitainer media; to control for possible leaching of estrogenic compounds from Cubitainers), or Milli-Q water (Milli-Q media; to control for any potential background estrogenic compounds in the LSW). The pH was adjusted to 7.3 before sterilizing 10 ml of the whole media samples by filtration through a 0.22-μm sterile syringe filter (Millex GP). The direct effluent media was serially diluted twofold in the Milli-Q media to produce concentrations ranging from 100% (1 ×) to 3.13% (0.0313 ×) direct effluent media.

T47D-KBluc assay

The T47D-KBluc cell line was developed by Wilson et al. [17], who also described basic approaches for maintaining the cell line. Cells were maintained in RPMI 1640 Media (GIBCO) supplemented with 1% Anti-Anti (antibiotic-antimycotic, GIBCO) and 10% fetal bovine serum (HyClone). Before conducting bioassays, the cells were cultured in RPMI 1640 media supplemented with 10% charcoal dextran–filtered fetal bovine serum without antibiotics and antimycotics for 7 d, reaching approximately 90% confluence. The cells were removed from culture flasks by a 15-min incubation in TrypLE™ Express (GIBCO) and transferred to assay media (RPMI supplemented with 5% charcoal dextran–filtered fetal bovine serum). They were then counted using a hemocytometer, diluted to 100,000 cells/ml in assay media, and seeded into clear-bottomed 96-well plates at 100 μl per well. Cells were allowed to attach for 1 h at room temperature before being transferred to a 37°C, 5% CO_2 incubator for approximately 24 h.

Extract exposures. The dilutions of SPE effluent extracts described previously were further diluted in assay media to yield final concentrations ranging from 1% (50× effluent) to 0.002% (0.1× effluent) or from 0.2% (10× effluent) to 0.002% (0.1× effluent) of extract, depending on the experiment. The

cells were dosed with 100 μl extract diluted in assay media or methanol solvent control, also diluted in assay media. Final methanol concentrations in the assay did not exceed 0.25%. All extract concentrations and controls were run in duplicate or triplicate on each plate, depending on space.

Direct effluent media exposures. For direct effluent media exposures, 100 μl effluent media dilutions, as described previously, were added to triplicate wells per concentration. Control wells received 100 μl fresh LSW media, Cubitainer LSW media, or Milli-Q media, each in triplicate.

Regardless of effluent sample preparation, an EE2 standard curve was run with each assay, and an E2 standard curve was run occasionally in addition to the EE2 curve. (See Supplementary Fig. S1 for a representative example of an EE2 and E2 standard curve.) Standard stock solutions were prepared in 100% ethanol. The EE2 standard curve was prepared for each assay, using a threefold serial dilution series in assay media, with the final concentrations ranging from 1.66 nM to 9.40×10^{-6} nM or from 0.667 nM to 3.76×10^{-6} nM, depending on the dilution in assay media. The 50% effective concentration (EC50) of EE2 was approximately 3 pM. An E2 standard curve with final concentrations ranging from 1.66 nM to 2.54×10^{-4} nM or from 0.667 nM to 1.02×10^{-4} nM, depending on the dilution in assay media, was prepared in addition to EE2 curves in three assays, two of which were conducted before the current study. The EC50 of E2 was approximately 5 to 6 pM. Standards were prepared so that solvent concentrations in each plate well were constant at 0.25% for the cell assay. Ethanol solvent controls were diluted in assay media and 100 μl added to the cells in duplicate or triplicate, depending on experimental design. All cell plating, dosing, and media preparation were conducted under a laminar flow hood to prevent bacterial and fungal contamination.

After exposure to standard, sample, or control solutions, cells were incubated for 16 to 24 h at 37°C under a 5% CO_2 atmosphere. After the incubation period, the exposure medium was removed, and the cells were subjected to a cytotoxicity assay (Invitrogen Live/Dead Viability/Cytotoxicity Kit), using the protocol developed by the manufacturer. After conducting the Live/Dead assay, the cells were washed with 25 μl/well 1× Dulbecco's phosphate-buffered saline (GIBCO) in preparation for the luminescence assay. The cells were lysed through a 30- to 60-min room temperature incubation period in 25 μl/well of Luciferase Cell Culture Lysis Reagent (Promega). Luminescence, in relative luminescence units, was determined using a luminometer (Biotek, Synergy 4; Gen5 Software) after the addition of 25 μl/well of reaction buffer (25 mM glycyglycine, 15 mM $MgCl_2$, 5 mM adenosine triphosphate, 0.1 mg/ml bovine serum albumin, pH 7.8) followed by 25 μl/well of luciferin (Promega), which were added directly to the plate by the luminometer as the plate was being read. Estrogenicity of the samples was calculated relative to the E2 or EE2 standard curves as described later.

In vivo exposures

Forty-eight-hour static renewal exposures with fathead minnows (*Pimephales promelas*) from an on-site culture facility at MED were conducted using the same effluent samples evaluated in the cell bioassays. The effluent exposure treatments consisted of an LSW control and three concentrations of effluent diluted in LSW (10, 50, and 100% effluent). In addition to the effluent exposures, a static renewal experiment was conducted with E2 to establish a positive control and calibration curve for the in vivo molecular endpoints. Dosing solutions for the E2 exposure were prepared in 100% ethanol. Each tank held 10 L LSW and was spiked with 500 μl dosing solution, yielding final E2 exposure concentrations of 0 (ethanol only, 0.005%), 1, 10, 100, and 250 ng E2/L. For each treatment within an experiment, two replicate tanks were used, and each tank held four sexually mature male fathead minnows. All tanks were aerated using air stones with constant flow, taking care to avoid splashing. Tanks were held in a 25 ± 1°C water bath with a 16:8 light:dark photoperiod, and fish were fed adult brine shrimp twice daily. One hundred percent of the treatment water was exchanged after 24 h. Fish were held in clean glass beakers filled with aerated day-old tank water while the water was exchanged. After 48 h of exposure, fish were anesthetized in buffered tricaine methanesulfonate MS-222 (Argent). Liver samples were collected, snap frozen in liquid N_2, and stored at −80°C until RNA was extracted. To prevent degradation by RNases or cross-contamination, dissection tools were cleaned with RNaseZap (Ambion) between each sample. All laboratory procedures involving animals were reviewed and approved by the U.S. Environmental Protection Agency MED Animal Care and Use Committee in accordance with the USA Animal Welfare Act and Interagency Research Animal Committee guidelines [23].

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction assays (QPCRs) were conducted to determine the relative abundance of VTG and ESR1 messenger RNA (mRNA) transcripts. Total RNA was extracted from the liver samples with TRI Reagent® (Sigma), using the manufacturer's protocol. Concentration and quality of the liver RNA samples were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). Total RNA from the effluent sample exposures on January 21, 2009 and March 25, 2009 was converted to complementary DNA (cDNA), using high-capacity cDNA reverse transcription kits (Applied Biosystems). All cDNA samples were diluted to 40 ng/μl, and Power SYBR Green PCR Master Mix (Applied Biosystems) was used to determine VTG and ESR1 expression relative to cDNA standards. The QPCRs were conducted as described by Ankley et al. [24], using the fathead minnow ESR1 primers (5′-CACCCACCAGCCCT-CAG-3′/5′-CACCTCACACAGACCAACAC-3′) described by Filby and Tyler [22] and the fathead minnow 5′ region VTG primers (5′-CACAATCCCAGCTCTGCGTGA-3′/5′-TGGCC-TCTGCAGCAATATCAT-3′) developed by Biales et al. [25]. Total RNA samples from the effluent exposures on April 13, 2009 and April 30, 2009, as well as the E2 exposure, were diluted to 10 ng/μl, and VTG and ESR1 relative abundance was determined using Power SYBR Green RNA-to-C_T one-step kits (Applied Biosystems), which included RT Enzyme Mix (125 ×) and Power SYBRGreen RT-PCR Mix (2 ×). For each 12 μl reaction, 2 μl (20 ng) RNA was combined with 200 nM forward and reverse primers, 3.86 μl nuclease-free water, 0.096 μl of RT Enzyme Mix (125 ×), and 6 μl Power SYBR Green RT-PCR Mix. The one-step QPCR amplification process was set up following the manufacturer's protocol. In all cases, relative transcript abundance of the target gene was quantified relative to a gene-specific cDNA standard curve (10-fold dilution series; 6× total concentrations). The gene-specific cDNA standards were prepared as described by Villeneuve et al. [26]; however, the second round of amplification for RNA standard preparation was not conducted. The same primers and cDNA standards were used for all QPCRs.

Statistical analyses

Statistical analyses of all the data were performed using GraphPad Prism 5 (GraphPad Software). Before statistical analyses, the QPCR gene expression data were converted to fold-change (log 10 scale) relative to the mean control value for each assay, to allow for comparisons between QPCRs. For the QPCR results, significant differences in mean gene expression compared with controls were determined by parametric one-way analysis of variance followed by Dunnett's multiple comparison test. Results in which $p < 0.05$ were considered significant.

Data from the in vitro T47D-KBluc assays were used to estimate the total estrogenic equivalents (EEQs) in the effluent samples, relative to E2 or EE2. The concentrations of the standards used in the cell assays were adjusted for dilutions in assay media (1:200 or 1:500) and log 10 transformed. The estrogenic activity of the test samples was interpolated by the least squares means procedure from a nonlinear sigmoidal dose–response curve fit to the relative luminescence units of the standards. The interpolated sample values were adjusted back to nanogram per liter concentrations and adjusted for sample dilutions in assay media (1:100 or 1:500). The EE2 EEQs for each effluent sample dilution series were determined using the EC50 of each respective estimate. To directly compare the estrogenicity of the effluent with the in vivo E2 exposure, the EE2 EEQs were converted to E2 EEQs based on three in vitro standard curve comparisons between these chemicals. Three separate assays, each containing E2 and EE2 standard curves, were conducted, and the EC50 values of each chemical were calculated for direct comparison between the two chemicals. Previously calculated EE2 EEQs were multiplied by the average ratio of E2 EC50 over EE2 EC50 to determine the E2 EEQs.

RESULTS

Comparison of in vitro sample preparation methods

In estimating the overall estrogenic activity of effluent, differences were evident between the T47D-KBluc cell assay results using the direct effluent method versus the SPE extracts. In initial work, a 10-fold dilution series of the August 5, 2008 methanol extract resulted in a non-monotonic concentration–response curve (Fig. 1a). As a result, E2 EEQ did not decrease in proportion to the degree of increased sample dilution. This made estimation of an EC50 and EEQs for the August effluent sample challenging. On December 19, 2008, the two sample preparation methods were directly compared for a single effluent sample. The SPE extract produced a non-monotonic concentration–response curve (Fig. 1a) such that the E2 EEQs for the 50× (data not shown) and 10× dilutions were less than those of the 1× dilution. An E2 EEQ could not be estimated for the 5× dilution because its relative luminescence units were greater than the maximum relative luminescence units of the E2 standard. However, from the 1× dilution to the 0.1× dilution, the E2 EEQs were approximately proportional with the degree of sample dilution. In contrast, the direct effluent sample preparation method produced a monotonic concentration–response such that a twofold dilution series resulted in an approximately proportional decrease in E2 EEQ concentrations for the December 19 effluent (Fig. 1b). The EC50 estimate of the effluent was 1.6 ng E2 EEQ/L. The direct effluent media approach was tested again on March 25, 2009, and a similar approximately proportional decrease in E2 EEQ concentrations with increasing dilution was achieved (Fig. 1b). Subsequent studies used the direct effluent media preparation method to evaluate total estrogenicity of the effluent samples.

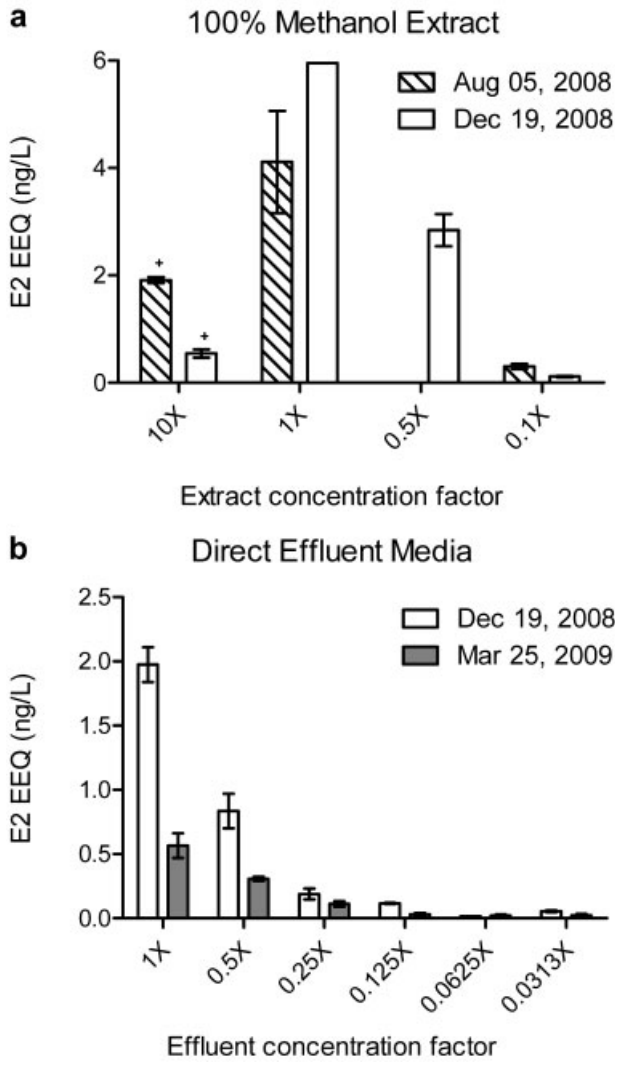

Fig. 1. Comparison of 100% methanol extracted sample dilutions and media prepared with effluent dilutions, expressed in 17β-estradiol estrogenic equivalents (E2 EEQs, ng/L) (mean ± SE). Standard error represents the replicate well variability within the cell assay. Direct data comparison for December 19, 2008 sample (white bars), and indirect data comparison for August 5, 2008 sample (striped bars) and March 25, 2009 sample (gray bars). (**a**) Dilutions of 100% methanol extracts. An E2 EEQ was not estimated for the 5× dilution from December 19, 2008, because the relative luminescence units were greater than the maximum relative luminescence units of the standard curve. + = Unreliable E2 EEQ estimate. (**b**) Direct effluent dilutions.

In vitro characterization of effluent estrogenicity

The estimated estrogenicity of the effluent, based on the direct effluent media, varied over the course of several months, with estimates of E2 EEQs ranging from 1.13 ng/L at the lowest in January to 2.00 ng/L at the highest in March (Fig. 2). No quantifiable levels of E2 EEQ were detected in the Milli-Q water, fresh LSW, or cubitainer LSW media controls (data not shown).

In vivo characterization

There was a concentration-dependent induction of ESR1 and VTG transcripts in male fathead minnows exposed to E2. The

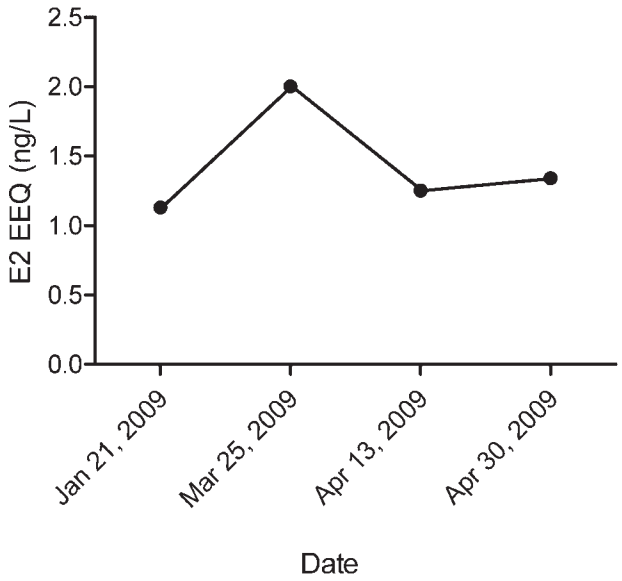

Fig. 2. Temporal variability of estrogenic activity of Western Lake Superior (Minnesota, USA) Sanitary District effluent, as estimated using the T47D-KBluc cell bioassay with direct effluent media. Expressed in 17β-estradiol estrogenic equivalents (E2 EEQ, ng/L) estimated based on the 50% effective concentration estimates for the sample.

ESR1 and VTG expression for the two highest treatment concentrations (100 and 250 ng E2/L) were both significantly ($p < 0.001$) elevated when compared with the corresponding solvent control (Fig. 3). No significant changes were seen in VTG and ESR1 expression in males exposed to 1 ng E2/L and 10 ng E2/L (Fig. 3b). The in vivo E2 exposure results provide evidence that the selected biomarker responses were inducible under the 48-h static renewal conditions, thereby supporting use of the same design for monitoring the effluent.

Three of the four in vivo effluent tests exhibited a concentration-dependent induction of estrogen-responsive genes VTG and ESR1, which was also observed in the in vivo E2 exposure. All of the 100% effluent treatments, except the effluent from March 25, 2009, resulted in a significant increase in transcript abundance of both VTG and ESR1 when compared with the LSW control (Fig. 3a). Only on April 30, 2009, did the 50% effluent treatment show a significant increase in expression of both genes over the LSW control. Vitellogenin expression from the 100% effluent treatment showed the greatest fold-change (log 10 scale) relative to the control (1.54 ± 0.43) on January 21, 2009, whereas ESR1 expression from the 100% effluent showed the greatest change (1.21 ± 0.09) on April 13, 2009 (Fig. 3a). No significant differences were evident from the LSW control in the relative transcript abundance of VTG and ESR1 in males exposed to 10% effluent (Fig. 3a).

DISCUSSION

Sample preparation methods

One goal of the current study was to compare the efficacy of two methods for preparing effluent for use in the T47D-KBluc bioassay to best quantify the total, integrated, estrogenicity of complex effluents. Final effluent from WWTPs is a complex matrix of many different chemicals. Consequently, some clean-up of the mixture is generally necessary before further analyses. Use of SPE columns is one method that has been used to isolate some of the most common known estrogenic compounds from effluents [3,6]. However, results of the current study indicate

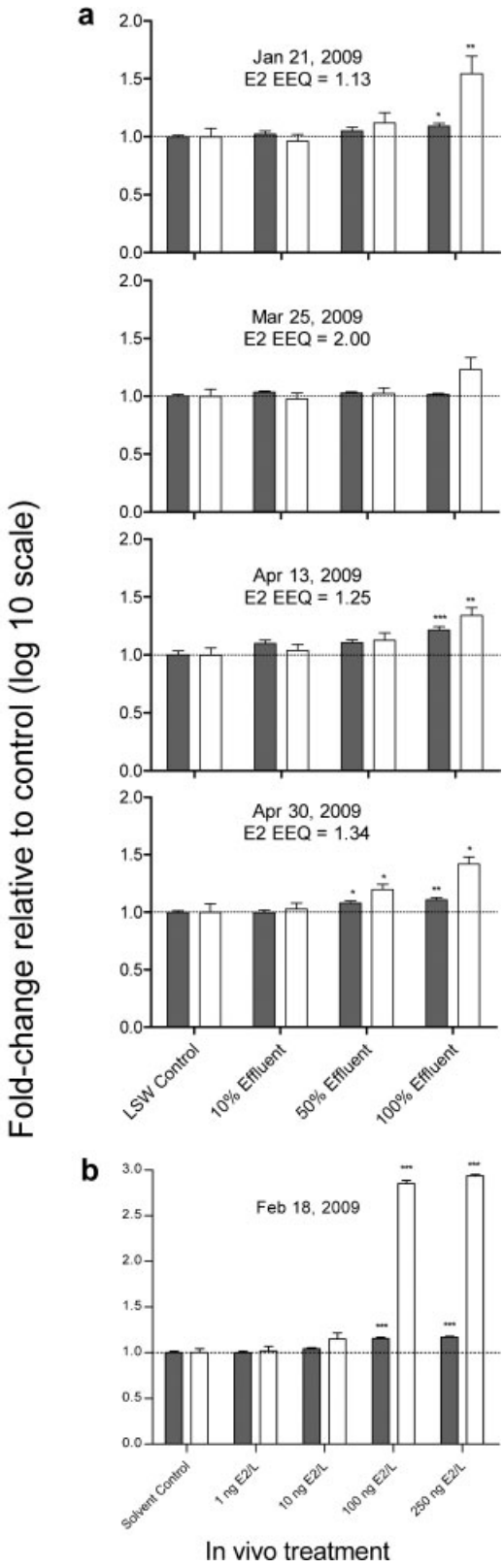

Fig. 3. Relative abundance of estrogen receptor α subunit (ESR1, gray bars) and vitellogenin (VTG, white bars) mRNA transcripts (mean ± standard error) of male fathead minnows exposed to (**a**) effluent samples collected on four different days in separate 48-h static renewal experiments or (**b**) 17β-estradiol (E2) in a 48-h static renewal experiment. Results are expressed as fold-change relative to control (log 10 scale). Estradiol estrogenic equivalents (E2 EEQ, ng/L) using the 50% effective concentration estimates are shown for each effluent sample. Statistically significant differences relative to controls are represented by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). LSW = Lake Superior Water.

that SPE elution with 100% methanol can result in interferences that lead to estimates of E2/EE2 EEQs that vary with sample dilutions (Fig. 1a). Based on results of the Live/Dead assay and use of solvent controls, these interferences are likely caused by the effluent extract and are not attributable to cytotoxicity or exposure to the solvent. For example, the sample from August 5, 2008 had estimated E2 EEQs of 1.9 ng E2 EEQ/L for the 10× extract, whereas the estimated E2 EEQs of the diluted 1× extract were actually approximately two times greater than the 10× extract. Further dilution of the effluent extract to 0.1× then resulted in a decrease in concentration to approximately 0.3 ng E2 EEQs/L (Fig. 1a). A similar inverted U-shaped, non-monotonic concentration–response was seen with the SPE extracts prepared on December 19, 2008. Estimated E2 EEQs for the 50× (data not shown) and 10× dilutions were less than those for the 1× dilution, and it was not possible to calculate an E2 EEQ for the 5× dilution. The non-monotonic nature of this concentration–response curve makes calculating a reliable EC50 estimate that can be used to estimate the overall estrogenicity of a sample difficult. Given that concentrated methanol extracts (e.g., 50 × , 10 × , 5 ×) were not significantly cytotoxic, the basis for the poor performance of the SPE method relative to consistent EEQ quantification is not entirely clear, especially given the complex nature of WWTP effluent. However, unless the issue can be rectified, the use of extracts will likely complicate quantification of the total estrogenicity of complex effluent using the T47D-KBluc cell bioassay.

In contrast, the direct effluent media preparation, which involves minimal clean-up of the effluent sample, provided more consistent estimates of estrogenic activity proportional to sample dilution. Dilution of the direct effluent media resulted in nearly linear monotonic concentration–response curves. The results of the current study suggest that the total estrogenicity of an effluent can be more reliably estimated based on the results from the direct effluent media rather than extracts. Extracts may still provide a semi-quantitative indication of the overall estrogenicity but would likely be subject to greater error because of apparent interferences. Although SPE extracts eluted in methanol would still be more amenable to bioassay-directed fractionation studies such as toxicity identification evaluations, direct effluent media sample preparation appears preferable for characterization of the overall activity of a whole effluent (e.g., for routine monitoring).

Temporal variation of effluent estrogenicity

Estrogenicity of WWTP effluents can fluctuate significantly over time [1,3,27,28]. As part of the current study, we were able to investigate this temporal variation. We collected wastewater effluent samples at the same time of day over the course of approximately three months. Although some variation was found in the absolute estimated estrogenicity among sampling dates, the range of E2 EEQs for the four samples we assessed were within a factor of 2, which is likely within the statistical precision of the assay. To attain a better portrait of variable effluent estrogenicity, collecting and analyzing additional samples from this WWTP throughout the full calendar year would be beneficial, given that seasonal change can be a contributing factor to variation in estrogenicity [27,28]. The relative composition (municipal versus industrial) of the influent wastewater processed by this particular WWTP could contribute to the temporal variation of its effluent's estrogenicity. The productivity of the mills fluctuates throughout the year, as influenced by economic factors or scheduled maintenance activities. Major fluctuations in mill productivity levels are typically communicated in advance and known by the WWTP staff. This variation would need to be considered in developing a monitoring strategy that employs the T47D-KBluc assay as a basis for evaluating effluent estrogenicity as a component of a toxicity identification evaluation. In fact, such variation could be intentionally leveraged to enhance the effectiveness of a toxicity identification evaluation investigation, because the municipal component remains relatively constant. However, these preliminary results suggest that the estrogenicity of the effluent examined in this study was fairly constant over a period of three months.

In vitro–in vivo comparison

The second major aim of the current study was to compare the results of the in vitro assays with those of an in vivo fish exposure to evaluate whether the recombinant human cell line–based assay was reasonably predictive of potential effects in fish. The findings of the in vivo fish assay supported the in vitro evidence that the effluent was estrogenic. Male fathead minnows exposed to the effluent dilutions showed concentration-dependent elevation of VTG and ESR1 mRNA transcripts. Males exposed to 100% effluent expressed a significant increase in VTG and ESR1 mRNA transcripts compared with the control males with three of the four effluent samples tested. These results lead us to conclude that ESR1 and VTG are both sensitive mRNA biomarkers of estrogen exposure in fish; however, the greater magnitude of induction of VTG makes it a more desirable mRNA biomarker for estrogen exposure screening. Based on cell bioassay results, the 100% effluent was estimated to contain 1 to 2 ng E2 EEQs/L. From this, it could be hypothesized that fish exposed to 1 or 10 ng E2/L would show VTG and ESR1 profiles similar to the estrogenic effluent. However, this was not the case. Instead, it appears that between 10 and 100 ng E2/L was needed to produce a significant induction of ESR1 and VTG transcription in vivo. Huggett et al. [11] found similar results, showing a 10-fold difference between the in vitro estimates of sample estrogenicity and in vivo results. In a study conducted by Thorpe et al. [29], EE2 was between 11 and 27 times more potent than E2 in fish. Our in vitro comparisons of E2 and EE2 suggested that EE2 is only roughly 1.7 times more potent than E2 in the T47D-KBluc cells. Hence, if EE2 were a dominant contributor to estrogenicity of the effluent examined in the current study, the discrepancy between the in vitro and in vivo relative potencies of E2 versus EE2 could explain significant increases in VTG and ESR1 transcript abundance in 100% effluent-exposed fish even though the E2 EEQs were estimated to be only 1 to 2 ng/L based on the in vitro assay. In addition, using an in vitro competitive estrogen receptor binding assay, Martinovic et al. [1] estimated the estrogenicity of WLSSD effluent between 4 and 33 ng E2 EEQ/L, suggesting that our T47D-KBluc assay results may be underestimating the estrogenicity of the effluent. Another possible explanation for this discrepancy may have to do with the potential for xenoestrogen bioaccumulation within the whole organism [11], which may cause a greater observed effect in gene expression changes relative to the lower in vitro estrogenicity estimate. Even though in the current study, the in vivo response was more sensitive to estrogen exposure than the T47D-KBluc cell assay, other similar studies have found the opposite results. For example, Pawlowski et al. [30] conducted three in vitro assays using differing cell types paired with in vivo fish exposures and determined that two of the three assays were more sensitive to estrogens than the in vivo exposures. The differences between the results of the Pawlowski et al. study and

the current study may be attributable to the fact that different cell lines were used for the in vitro assays [30].

In conclusion, the direct effluent method of sample preparation examined in the current study proved an effective technique for characterizing estrogenic activity in WWTP effluent, and the T47D-KBluc cell bioassay successfully predicted the molecular (estrogenic) responses in fish. However, further investigation may be necessary to understand the apparent discrepancy in the potency estimates based on comparison of the in vitro versus the in vivo responses relative to an E2 standard. Overall, the T47D-KBluc cell assay appears to have utility for effluent monitoring applications and also may, in addition to effluent characterization, be effective in evaluating spatial dilution of estrogenic activity relative to the site of effluent discharge. With the development of appropriate fractionation methods, this assay also should be practical for bioassay-directed fractionation.

SUPPLEMENTAL DATA

Fig. S1. (42 KB PDF)

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