Comparison of in Vitro and in Vivo Bioassays for Estrogenicity in Effluent from North American Municipal Wastewater Facilities

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Attempts to better understand causal factors affecting estrogenicity in municipal wastewater have primarily focused on analytical evaluation of specific chemical estrogens and the use of estrogen receptor (ER) based in vitro assays. To compare analytical, in vitro, and in vivo assays for estrogenicity, wastewater from four New York and one Texas municipal wastewater facilities was evaluated for estrogenic activity using the yeast estrogen screen assay (YES) and an in vivo fish vitellogenin (VTG) assay. Estrogenic activity, as measured by the YES assay, was observed in methanol and/or methylene chloride eluents from C18 extracts in two of the New York treatment facilities and the Texas facility. Estradiol equivalents for the YES assay data ranged from ≤1 to 15 ng/l. Male Japanese medaka (Oryzias latipes) were then exposed for 7 days to solvent extracts from the New York-Red Hook facility and the Texas facility. Hepatic and plasma vitellogenin were induced in medaka after exposure to the methanol eluent from the New York facility, even though the YES assay indicated that both the methanol and methylene chloride eluents from C18 extracts in two of the New York treatment facilities and the Texas facility. Estradiol equivalents for the YES assay data ranged from ≤1 to 15 ng/l. Male Japanese medaka (Oryzias latipes) were then exposed for 7 days to solvent extracts from the New York-Red Hook facility and the Texas facility. Hepatic and plasma vitellogenin were induced in medaka after exposure to the methanol eluent from the New York facility, even though the YES assay indicated that both the methanol and methylene chloride eluents from C18 extracts in two of the New York treatment facilities and the Texas facility. Estradiol equivalents for the YES assay data ranged from ≤1 to 15 ng/l. Male Japanese medaka (Oryzias latipes) were then exposed for 7 days to solvent extracts from the New York-Red Hook facility and the Texas facility. Hepatic and plasma vitellogenin were induced in medaka after exposure to the methanol eluent from the New York facility, even though the YES assay indicated that both the methanol and methylene chloride eluents from C18 extracts in two of the New York treatment facilities and the Texas facility. Estradiol equivalents for the YES assay data ranged from ≤1 to 15 ng/l. Male Japanese medaka (Oryzias latipes) were then exposed for 7 days to solvent extracts from the New York-Red Hook facility and the Texas facility. Hepatic and plasma vitellogenin were induced in medaka after exposure to the methanol eluent from the Texas facility, plasma VTG induction was observed in both the methanol and methylene chloride eluents. In vivo estrogenic activity was nearly 10-fold greater than YES activity indicating the presence of nonestrogen receptor ligands that elicit estrogenic effects in fish through indirect mechanisms. The sole use of in vitro assays to screen for estrogenicity may underestimate estrogenic potential of wastewater.

Key Words: YES; wastewater effluent; estrogenic activity; vitellogenin.

Steroid-mimicking compounds have been identified in surface waters throughout Europe and North America (Kolpin et al., 2002; Routledge et al., 1998; Snyder et al., 2001; Ternes et al., 1999a). Estrogenic compounds derived from human wastewater are the most frequently observed steroids in surface waters and have been implicated as causative agents for widespread reproductive dysfunction in aquatic fish species in the U.K. (Jobling et al., 1998). Estrogen receptor based bioassays were instrumental in identifying several compounds in wastewater effluent as causative agents following various chromatographic steps similar to Toxicity Identification Evaluations (Desbrow et al., 1998). Endogenous estrogens such as 17β-estradiol (represented as estradiol throughout), estrone, and estril as well as the synthetic estrogen, ethynyl estradiol were frequently observed in wastewater from the U.K. presumably derived from pharmaceutical use or naturally excreted by humans. Unfortunately, compounds not binding the estrogen receptor were ignored and not identified in any of these studies.

Previous studies in our laboratory have indicated a significant discrepancy between estrogenic activity of wastewater effluents measured using in vitro and in vivo dose-response estrogen equivalents (Thompson et al., 2000; Tilton et al., in press). Indeed when analytical evaluation of these wastewaters was conducted, quantities of natural estrogens were comparable to equivalency values calculated from in vitro assays using ER-based reporter systems. However, when vitellogenic responses were determined, significantly higher equivalency values were observed, in some cases approaching a 10-fold difference (Thompson et al., 2000).

To determine the relative contributions of ER agonists and indirectly acting estrogenic compounds, the following study was conducted in which in vitro and in vivo bioassays were utilized in an evaluation of wastewater effluents from two treatment facilities. A 10-fold difference was again noted in overall estrogenic response between ER and in vivo bioassays suggesting the occurrence of other estrogenic compounds that have not yet been identified possessing target sites alternative to the ER.

MATERIALS AND METHODS

Sampling and extraction protocol. As previous studies in New York City (NYC) indicated significant estrogenic activity in wastewater, initial analytical...
studies were performed to measure concentrations of specific estrogenic substances such as nonylphenol (NP), octylphenol (OP), estrene, 17β-estradiol, and ethynylestradiol. Wastewater treatment samples were collected on 21 October 2000 and 1 February 2001 from Newtown Creek (NC; partial secondary treatment; 310 million gallons per day [MGD] average capacity), Red Hook (RH; full secondary treatment; 60 MGD average capacity) and 26th Ward (26th; full secondary treatment; 85 MGD average capacity) wastewater treatment facilities on Long Island, NY. Wastewater samples from Denton were collected on 15 May 2001 from a wastewater treatment facility using full secondary treatment with an average capacity of 11 MGD. Samples were collected in acid- and solvent-rinsed 20-l glass containers at similar times of the day each sampling date. Effluent was first passed through Millipore glass fiber prefilters. For analysis of estrene, 17β-estradiol, and ethynylestradiol, one liter of effluent was passed through Empore™ SDB-XC extraction disks, which were previously conditioned with acetone, methanol, and water (Belfroid et al., 1999). For NP/OP analysis, effluent was adjusted to pH 2 and passed through Empore™ C18 extraction disks, which were previously conditioned with acetone, methylene chloride, and hexane (Bakerbond Application Notes and Ferguson et al., 2000). Samples were frozen at –80°C until analysis.

Analytical chemistry. Estrogen analysis followed methods outlined by Belfroid et al. (1999) and Ternes et al. (1999b). Compounds were eluted off the SDB-XC extraction disks with two 15 ml additions of methanol. The methanol eluents were then combined and mixed with 1 g hot sodium sulfate, which under nitrogen was reduced to 5 ml. The extract was transferred to graduated concentrator tubes and reduced to dryness. Samples were derivatized with 50 μl of MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide)/TMSI (trimethylsilylimidazole)/DTE (dithioerytrol) (1000:2:2 mixture). After 30 min at 60°C, samples were evaporated to dryness, reconstituted in 100 μl of hexane and transferred to gas chromatograph vials. Samples were analyzed on a Hewlett Packard 6890 series gas chromatograph (GC) with a Hewlett Packard 5973 mass selective detector. A J&W DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) with a 0.9 ml gas flow rate was utilized in this study. The temperature profile for each GC sample run was 160°C for 1 min increased to 290°C at 10°C/min and held at 290°C for 10 min (24 min total run time). Samples were analyzed in selective ion monitoring (SIM) with ions 425, 342, and 416 m/z utilized for monitoring ethynylestradiol, 17β-estradiol, and estrone, respectively. Quantitation of estrogens was accomplished utilizing a three-point calibration curve. Detection limits for all compounds were ≤1 ng/l. Recovery of spiked estradiol in water was used as a model for estrone and ethynylestradiol and was 82%.

NP and OP were determined according to methods outlined by Ferguson et al. (2001a). Two 15 ml aliquots of acetone were utilized for elution of compounds from C18 extraction disks. Acetone eluents were combined and reduced in volume to 500 μl under nitrogen. The final volumes were transferred to autosampler vials and analyzed on a Finnigan liquid chromatographic instrument with mass selective detection. Samples were analyzed in SIM mode with 205 and 219 m/z representing OP and NP, respectively. Quantitation of analytes was accomplished through the use of naphthal as an internal standard. Detection limits were set at 1 μg/l for both compounds. Recovery of spiked NP and OP in deionized water was 71 and 82%, respectively.

Yeast estrogen screen (YES) assay. Since initial analytical results from the NYC treatment plants did not correspond to previous in vivo estimates of estrogenic activity (Thompson et al., 2000), a subsequent in vitro assay measuring estrogen receptor ligands (e.g., the YES assay) was performed according to methods prescribed by Desbrow et al. (1998). Samples for these analyses were collected from the three NY treatment facilities on 21 October 2000 and 1 February 2001. Denton samples were collected on 15 May 2001. In this assay, the human estrogen receptor (hER) and estrogen response elements (hERE) are integrated into Saccharomyces cerevisiae (yeast do not normally express the estrogen receptor). Since the ERE is linked to the β-galactosidase reporter gene, a colorimetric change at 405 nm is observed when an ER ligand binds the receptor and is translocated to the ERE.

To initially separate and characterize the estrogenic activity of wastewater effluents, hydrophobic compounds were extracted from the wastewater with Empore™ C18 disks and then eluted sequentially with methanol, methylene chloride, and hexane (Desbrow et al., 1998). Studies using spiked concentrations of effluent indicated no significant difference in estradiol or NP retention or recovery between Empore SDB-XC or C18 disks. Each solvent eluent was then reduced to dryness and reconstituted in 300 μl ethanol. Growth media (5 ml) was then inoculated with either ERE (estrogen response element that serves as a negative control) or ERE/ER (estrogen response element and estrogen receptor) cells. After overnight incubation at 30°C, cells were diluted to an optical density of 0.057 (determined at 630 nm). The solvent eluents (100 μl) were subsequently incubated with 700 μl of ER/ERE cells, ERE cells, or ETOH. After 24 h incubation at 30°C, 100 μl of each suspension was added to a 96 well microtiter plate with 400 μl of the chromogenic substrate (ONPG; Sigma Chemical Co., St. Louis, MO). The plates were incubated until the color developed 2–3 days at which point optical density was determined at 405 nm. Optical density readings were corrected for untreated ETOH controls, turbidity, and ERE interactions as follows: OD = ER/ERE (Test405 nm − (Test 630 nm − Con405 nm) − Con630 nm) − ERE (Test405 nm − (Test 630 nm − Con430 nm) − Con630 nm) (Payne et al., 2000). Detection limits were ≤1 ng estradiol/l. Each measurement was made in duplicate and a dose response curve was constructed to estimate estradiol equivalents of wastewater eluents (Fig. 1). To determine recovery extraction recoveries, water was spiked with 10 μg/l of estradiol and NP. Upon triplicate analyses, 71 ± 10% of the NP and 82 ± 18% of the estradiol eluted in the MeOH eluent. The compounds were not detected in the MeCl or hexane eluents.

In vivo medaka screening bioassay. To measure other indirect acting and direct (ER) acting estrogenic activity, solvent eluents from C18-extracted effluents were screened using an in vivo medaka model (Nimrod and Benson, 1998; Thompson et al., 2000). Samples from only the Red Hook treatment plant (NYC) collected on 1 February 2001 and the Denton facility (15 May 2001) were evaluated. Adult Japanese medaka, Oryzias latipes, were selected randomly from a stock culture maintained at the University of Mississippi for approximately seven years. All adult fish in this study were fed TetraMin flakes and brine shrimp in the morning and brine shrimp only in the afternoon. Each exposure chamber contained 800 ml of a balanced salt solution (BSS) reconstituted in Nanopure filtered water with two adult male fish, three replicates per treatment. Animals were aequously treated with 50 μl of extracts added to the 800 ml BSS water for seven consecutive days with complete 24 h static water renewal.

FIG. 1. Concentration response curve of estradiol in the YES assay. Each value represents the mean of two replicates.
For the New York experiments, livers and plasma from fish were collected and analyzed for vitellogenin (VTG) by Western blot as described previously (Nimrod and Benson, 1998; Thompson et al., 2000) using a polyclonal antibody for striped bass vitellogenin provided by Dr. Nancy Denslow. This antibody has been previously shown to selectively recognize medaka vitellogenin by Western analyses (Nimrod and Benson, 1997). Only plasma was evaluated from fish exposed to Texas wastewater.

Five replicates per dose were used to construct a 17β-estradiol dose-response curve (Fig. 2). One treatment group only received 50 μl of an ethanol carrier. This was considered the control treatment group. Hepatic vitellogenin bands were digitized and dose response curves were constructed using a ratio of the estradiol response to the control response. Estradiol equivalents were determined by linear extrapolation from the curve as previously described (Thompson et al., 2000). Estradiol equivalents were not calculated for plasma vitellogenin.

Statistical comparisons. Parametric ANOVA was utilized to distinguish treatment differences in plasma vitellogenin following Bartlett’s homogeneity test for normality. Bonferroni’s test was used to determine significance (p ≤ 0.05).

RESULTS

Measurable concentrations of estradiol and estrone were observed in wastewater effluents sampled from New York (Table 1). Estrone concentrations ranged from below detection (≤1 ng/l) to 42 ng/l, and estradiol concentrations varied from no detection to 20 ng/l. NP was identified in each of the six New York samples at concentrations ranging from 12 to 79 μg/l (Table 1). OP and ethynylestradiol were not identified in any of the samples.

Wastewater compounds removed by C18 extraction and subsequent solvent elution were evaluated by YES and in vivo fish assays. The majority of estradiol equivalents determined by YES were consistently in the methanol eluents of the C18 extracted components of the Red Hook and Denton effluents. Approximately two-fold higher activity was observed in the methanol eluent of the Red Hook effluent than in the methylene chloride eluent, and no significant activity was observed in the hexane extract. A similar relationship was observed in the Denton wastewater extracts where 100% of the estrogenic activity was observed in the methanol eluent and no activity observed in either the methylene chloride or hexane eluents.

When Japanese medaka were exposed to the solvent eluents from the C18-sorbed disks which filtered wastewater from the Red Hook plant, hepatic vitellogenin expression was observed in the methanol eluent with lesser responses in the methylene chloride and hexane eluents. The methanol eluent possessed 88% of the total in vivo vitellogenin-derived estrogenic activity of the Red Hook wastewater. Approximately 8 and 4% of the estrogenic activity was in the methylene chloride, and hexane eluents, respectively (Table 2). The Denton extracts caused significant plasma vitellogenin expression in both the methanol (41%) and methylene chloride (47%) eluents with measurable activity also present in the hexane eluent (12%; Fig. 3). In contrast to the YES results, there were approximately equal concentrations of expression between the methanol and meth-

### TABLE 1

<table>
<thead>
<tr>
<th>Date/Location</th>
<th>YES</th>
<th>Estradiol</th>
<th>Ethynylestradiol</th>
<th>Estrone</th>
<th>NP*</th>
<th>OP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 October 2000</td>
<td></td>
<td>9.0</td>
<td>20</td>
<td>&lt;1</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>1 February 2001</td>
<td></td>
<td>11</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>4</td>
<td>43</td>
</tr>
<tr>
<td>15 May 2001</td>
<td></td>
<td>7</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Note. For YES, values represent the average of duplicate analyses; for all others, values represent single sample determinations. For Denton, values for all columns represent the average of duplicate analyses. ND, no detection; —, not measured.

*Values are in μg/l.

### TABLE 2

<table>
<thead>
<tr>
<th>Location</th>
<th>Methanol</th>
<th>Methylene chloride</th>
<th>Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Hook</td>
<td>YES*</td>
<td>6–7</td>
<td>3–4</td>
</tr>
<tr>
<td>VTG*</td>
<td>225 ± 40</td>
<td>20 ± 1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Denton</td>
<td>YES</td>
<td>7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>VTG*</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Note. ND, not detected; —, not determined.

*Values represent the range of two sampling dates.

*Values represent the mean of three replicate assays ± SD.
ethylene chloride eluents utilizing the in vivo bioassay for the Denton effluent. Significant increases in plasma vitellogenin were observed only in the methanol eluent from New York (Fig. 4).

**DISCUSSION**

The overall aims of this study were to compare in vitro and in vivo bioassays of two wastewater effluents from North America. Although a recent study compared in vivo and in vitro potency of various estrogens in the laboratory (Legler et al., 2002), most groups have utilized in vitro methods of estrogenic activity to evaluate surface water or fractionated effluents of municipal wastewater for the occurrence of estrogenic agents (Desbrow et al., 1998; Sheahan et al., 2002b; Snyder et al., 2001). In contrast, in vivo assays have been utilized primarily in the field or in laboratory studies with specific compounds or wastewater effluents (Folmar et al., 1996; Harries et al., 1996, 1997, 1999; Metcalfe et al., 2001). Although several studies have compared in vitro and in vivo bioassays for estrogenic activity (Beresford et al., 2000; Metcalfe et al., 2001), there have not been any published reports utilizing in vivo assays of estrogenicity to identify causative agents in wastewater effluent.

Analytical evaluations of the NY effluents in the fall and winter for common estrogens such as estradiol and estrone indicated values consistent with those which have been previously observed in wastewater. The highest concentration of estradiol (20 ng/l) from Red Hook was higher than mean concentrations (low ng/l) observed in other effluents from the U.S., Canada, Brazil, and Europe (Desbrow et al., 1998; Huang and Sedlak, 2001; Korner et al., 2000; Snyder et al., 2001; Ternes et al., 1999b), but lower than values in certain U.K. facilities (88 ng/l; Rodgers-Gray et al., 2001) and those observed recently in the U.S. (hundreds ng/l; Kolpin et al., 2002).

Earlier studies on the 26th Ward whole effluent observed values of estradiol at 6.4 ng/l and estrone at 17.6 ng/l (Ferguson et al., 2001b). Synder et al. (1999) found ≤4 ng/l estradiol in municipal wastewater effluent samples from Michigan, whereas ethinylestradiol was measured in the pg/l concentrations. In Lake Mead, estradiol concentrations ranged from below detection to 2.6 ng/l (Snyder et al., 1999). Desbrow et al. (1998) measured maximum estrone and estradiol concentrations in U.K. municipal effluent of 76 and 48 ng/l, respectively. In Canadian municipal effluent, estradiol was ≤64 ng/l (Ternes et al., 1999b). Estradiol, ethinylestradiol, and estrone were identified in German effluents at maximum concentrations of 3, 15, and 70 ng/l (Ternes et al., 1999b). Estrone was consistently identified in effluents from the Netherlands (≤47 ng/l; Belfroid et al., 1999) and the U.S. (Kolpin et al., 2002).

Consequently, extremely variable concentrations of natural and synthetic estrogens are frequently observed in surface waters throughout the world that do not consistently correspond to season or wastewater treatment. As these agents represent a fraction of the unknown chemicals in wastewater effluent, it is important to determine if other estrogenic compounds can be identified.

In vitro assays such as the YES assay have been utilized in effluent fractionation studies to help identify estrogenic agents in environmental media. This method utilizes the biological response of estrogen receptor activation which can be measured in equivalent values of the natural ligand of the receptor, estradiol. Estradiol equivalents derived from a concentration-response curve of the YES assay for the solvent eluents of C18 extractable compounds were approximately half of the analytical values for estradiol in the Red Hook wastewater. Although Empore SDB-XC disks with methanol/acetone elution were initially used for estrone and estradiol analytical measurements, similar recovery values were obtained using Empore C18 disks with exclusive methanol elution. Therefore, differ-

![FIG. 3. Plasma vitellogenin expression in medaka following exposure to solvent eluents from the Denton, Texas, wastewater. Each value represents the mean of three assay replicates ± SD.](http://toxsci.oxfordjournals.org/)

![FIG. 4. Plasma vitellogenin expression in medaka following exposure to solvent eluents from the Red Hook, New York, wastewater. Each value represents the mean of three assay replicates ± SD.](http://toxsci.oxfordjournals.org/)
ences in extraction and cleanup are not likely responsible for the differences between observed between YES and the two most potent estrogens measured in this effluent. A recent study in the Netherlands demonstrated that there was 5–10 times greater predicted estrogenic activity through in vitro systems than was actually measured (Murk et al., 2002). In contrast, lower in vitro activity versus estrogen concentrations was also observed in Nevada wastewater evaluations (Snyder et al., 2001). The lower YES activity compared to the analytical data indicates either interference by other compounds in the extract with the YES assay (Snyder et al., 2001), and/or a lack of bioavailability for, at least, estradiol to interact with the estrogen receptor in the yeast. Earlier studies comparing analytical measurements of estrogens and in vitro estrogenic activity of wastewater obtained from a predominantly domestic treatment facility showed nearly identical values (Snyder et al., 2001; Tilton et al., 2002). Complexation with dissolved organic material in the wastewater may be a possible explanation for the impaired absorption by the yeast. Interaction with colloidal material of wastewater has been previously observed with estradiol and thus, may explain the discrepancies between analytical values of estrogens and the in vitro assay (Bowman et al., 2002; Johnson and Sumpter, 2001; Williams et al., 1999).

Discrepancies were also observed between YES and the in vivo bioassay (medaka VTG) for estrogenicity in NYC and Texas effluents sampled on the same dates. Significantly higher estrogenic activity was observed using the medaka assay compared to the YES assay and analytical measurements of estrogens for both effluents. It is not uncommon for in vitro estrogenic activity to differ from in vivo (Metcalfe et al., 2001). Schreurs et al. (2002) demonstrated in vitro binding of six xenoestrogens to the ER, but these compounds failed to induce vitellogenin in zebrafish. In addition to the already mentioned possibility of YES suppression by other compounds, two other potential explanations for enhanced in vitro activity are the enhanced bioavailability/absorption of agents by the fish relative to the yeast, and/or the occurrence of non-ER ligands, which elicit estrogenic activity through indirect mechanisms. As vitellogenin synthesis is directly controlled by hepatic ER activation, persistence of any estrogen or removal from plasma binding proteins would perpetuate the estrogen response. Potential mechanisms of maintaining elevated estrogen concentrations would include induction of estrogen synthesis (either indirectly or through aromatase augmentation), or removal of estrogens from binding proteins or reductions of estrogen elimination through inhibition of biotransformation or excretion pathways. In addition, alteration of feedback loops by a contaminant in which estradiol plays a role may also be affected (i.e., antiandrogenicity). Future studies are necessary to initially determine the specific agent(s) responsible for these in vivo effects.

Significant differences in the estrogenic activity of the solvent eluents were observed between in vitro and in vivo assays. Generally, most of the in vitro activity was associated with the methanol eluent of the C18 extracted wastewater. The methanol eluent appeared to have a greater effect on estrogenic activity as measured by hepatic vitellogenin (88% of total) compared to plasma concentrations (45%) of the New York effluent. These data suggest agent(s) that may be specifically transported to the liver. However, the identity of these agent(s) remains a mystery. Estradiol was predominantly found in the methanol eluent, which is consistent with previous studies (Snyder et al., 1999, 2001). Indeed, the analytical method for estradiol and estrone utilized in this study followed a similar sample extraction procedure. However, as estrogens were not measured in this eluent (all was used for in vitro and in vivo assays), the actual concentrations of known endogenous or synthetic estrogens in this methanol eluent are uncertain.

In the Red Hook (NYC) effluent, consistent estrogenic activity as measured by YES and medaka VTG was also observed in the methylene chloride eluent of the effluent. In previous studies, this eluent contained alkylphenol compounds including metabolites such as NP (Snyder et al., 1999, 2001). In contrast, NP was found exclusively in the methanol eluent in the current study, which is consistent with other investigations that utilized a somewhat different extraction method but similar solvents (Ferguson et al., 2000, 2001a). Total concentrations of NP in the Red Hook effluent were approximately 28 μg/l, which exceed threshold concentrations for VTG induction under the conditions of this assay (20 μg/l; Islinger et al., 2002). Snyder et al. (1999) measured NP in municipal effluents at concentrations ≤33 μg/l, while OP was ≤673 ng/l. Previous studies of the Denton, TX, effluent have reported mean NP values from below detection to 2.5 μg/l (Hemming et al., 2001). In U.K. municipal effluents, maximum concentrations of NP and OP were 2.8 and 0.28 μg/l, respectively (Desbrow et al., 1998). Recent studies have shown that wastewater effluents omitting alkylphenolic compounds from the influent prior to treatment had significantly reduced estrogenic activity compared to wastewater effluents having significant alkylphenolic constituents (Sheahan et al., 2002a). Alkylphenolic ethoxylate (APEO) metabolite concentrations have been measured in the range of 70 to greater than 500 μg/l in effluents from four NYC plants (Todorov, unpublished data). Recent studies indicate APEOs are possibly more likely to be causing estrogenic responses in juvenile striped bass exposed to those sewage effluents than are the steroid estrogens, which are found at significantly less concentrations (10,000–30,000 lower) than the APEOs (Todorov et al., 2002). Thus, although alkylphenols as well as estradiol may be causative agents in the methanol eluent, the potential agents in the methylene chloride eluent are unknown.

Although estrogenic activity as measured by the YES and in vivo assays was relatively similar between the Red Hook and Denton treatment plants, higher in vivo activity of the methylene chloride eluent was observed in animals exposed to Denton wastewater. In contrast, no YES activity was observed in the
methylene chloride eluent from Denton, indicating a compound(s) not absorbed by yeast or an ER-inactive agent(s). As neither NP nor estradiol elutes from the filter with methylene chloride, the causative agents are unknown. Given that 58% of the Denton treatment plant receives wastewater from industrial sources, whereas most of the New York effluent is from domestic sources, the identity of the agent(s) may be of an industrial nature in the methylene chloride eluent. Studies are currently underway to hopefully identify potential agent(s).

Hexane eluents were not active in any of the YES assays but showed minor estrogenic activity in vitro. A similar lack of in vitro response was observed in wastewaters of Nevada (Snyder et al., 2001). Compounds eluting in this eluent would likely be legacy persistent organic pollutants (POPs) such as organochlorine or potentially polyaromatic hydrocarbons typically found in industrial effluents. Like NPs, these compounds have limited ER potency and act indirectly as estrogens or antiandrogens (Kelce et al., 1995; Khan and Thomas, 1996). Although these compounds make up only 10–12% of the in vivo estrogenic activity, they should not be ruled out as causative agents, as they may act in concert with other direct acting estrogenic compounds.

In summary, wastewater effluents from several North American treatment facilities were evaluated analytically for specific target estrogens as well as with in vitro and in vivo bioassays. In vitro assays severely underestimated the estrogenic activity of these wastewater effluents. Ongoing studies will be carried out to identify causative agents of in vivo estrogenicity in a mass balance approach to determine if compounds are acting in concert or independently of one another as environmental estrogens.

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