Effect of chlorination on estrogenicity in chlorinated treated effluent

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Abstract Effluents from sewage treatment plants can be discharged into rivers with estrogenic contaminants at levels sufficient to induce adverse reproductive and fertility developments in humans and wildlife. Of great concern in recent years are the estrogenic activities of chlorinated by-products (CBPs) in effluents. Simplified cell proliferation tests using the human estrogen receptor-positive MCF-7 ATCC breast cancer cells (E-SCREEN) were performed to investigate the influence of chlorination on the estrogenicity in effluents. It was found that the increase of chlorine dosages from 0 mg/L to 4 mg/L led to the decrease of total content of estrogenic activity: the 17ß-estradiol equivalent concentration (EEQ) from 22.40 to 8.35 ng/L. However, the increase of contact time from 10–30 minutes increased EEQ from 14.04 to 29.97 ng/L. Furthermore, increasing TOC level using humic acid from 5 mg/L to 15 mg/L in effluents correspondingly resulted in an increase of EEQ from 19.69–27.20 ng/L; it was thus confirmed that chlorination of humic acid could produce estrogenic by-products.

Keywords Chlorinated by-products; chlorination; E-SCREEN; estrogenic activity

Introduction
In recent years there have been many strong retrospective observations regarding adverse reproductive and fertility developments in humans and wildlife. In humans, for example, there have been increasing documented cases of testicular and female breast cancer, as well as increasing number of reports suggesting the degradation in semen quality, cryptorchidism (undescended testes), hypospadias (malformation of the penis) and polycystic ovaries (Harrison et al., 1997). Amongst wildlife, there have been many reports of dwindling populations, increases in cancers, reduced reproductive function and disrupted development of the immune and nervous system (Jimenez, 1997). There is now unequivocal evidence that a wide variety of chemicals, referred to as ‘endocrine disruptors’, capable of mimicking or antagonizing the effects of endogenous hormones are present in the environment and are contributing to these observed effects in humans and wildlife. Of great concern in recent years are the possible estrogenic activities of chlorinated by-products (CBPs), especially when taking into account the widespread use of chlorination as a disinfecting agent in modern drinking and wastewater treatment plants.

Currently, little is known about the effects of chlorination on estrogencity. Some reports suggest that chlorination destroys estrogenic activities of specific known estrogenic chemicals, through the strong oxidizing power of chlorine which alters the estrogenic structures of endocrine disrupting compounds (Hu et al., 2002). Other reports recommend an increase in estrogenic activities, suggesting that the production of chlorinated by-products may have caused the production of compounds which are able to induce estrogenic responses and elicit endocrinial or anti-endocrinial effects (Itoh et al., 2000; Yuan et al., 2003). It is feasible that the final estrogenic activity of a chlorinated water sample may be a combination of both scenarios. The problem is compounded by
the complex reaction kinetics of chlorine when it reacts with organic materials. In addition, different species or concentrations of chlorinated by-products are produced under different chlorination conditions. Furthermore, a large portion of the chlorinated by-products consists of unknown organic halogens, which makes the study of their estrogenic activity more difficult. Thus, more study is needed in this area before any conclusion can be made.

E-SCREEN was chosen as the biological screening assay to assess estrogenic activity in this study. This is because the proliferation test with ER-positive human MCF-7 breast cancer cells is a powerful tool for the sensitive quantitative determination of overall estrogenic activity in municipal wastewater after solid phase extraction without any clean up step (Körner et al., 1999). Using E-SCREEN assay, the present study investigates the influence of chlorination conditions, such as chlorine dosage, temperature, pH, and contact time, as well as the total organic carbon (TOC) concentrations, on the estrogenicity of the chlorinated samples.

Methods
Chemicals
17β-estradiol (E2), HPLC grade ethanol, acetone and methanol, reagent grade sodium hypochlorite, commercial grade humic acid, Bisphenol-A (97%), 4-tert-butyphenol (99%), 4-chloro-3-methylphenol (99%) and 4-chloro-2-methylphenol (97%), were obtained from Sigma-Aldrich (St. Louis, MO). E2, chloroform and TCA were stored in ethanol as 1 mM solutions at −20°C and diluted with experimental medium before use in assays. Sterile and pretested Dimethylsulfoxide (DMSO) was obtained from ATCC (Manassas, VA). 500 mg Strata C18 cartridges were from Phenomenex (Torrance, CA). AR grade hydrochloric acid (HCl, 37%) was from Merck Inc. (Haar, Germany).

Sampling of sewage effluent
Prechlorinated secondary effluents (after final settling tank) from a local water reclamation plant were collected in 1 L brown glass bottles. Immediately upon arrival in the laboratory, the samples were stored at 4°C until extraction. Samples were used within a few days to avoid the biodegradation of estrogen compounds.

Chlorination
Chlorination was completed in dark Duran bottles placed in a water bath to maintain a temperature of 20°C and pH 7.0, adjusted using hydrochloric acid (HCl) for a preset contact time. Sodium hypochlorite (NaOCl) of strength 5% was used as the chlorine source. The level of combined residual chlorine was measured using a colorimeter (Hach DR1890). Chlorination was quenched using stoichiometric amount of aqueous sodium thiosulphate (Na2S2O3).

Extraction of sewage effluent
Solid Phase Extraction on columns with 500 mg strata C18 and Visiprep TM Vacuum Manifolds (Sigma-Aldrich, St. Louis, MO) was applied as a quick extraction method requiring very low amounts of solvent. Before extraction was performed, 1 L samples were filtered through Whatman 0.45 µm glass filter paper. pH was then adjusted using 1.0 N hydrochloric acid (HCl) to pH 3.0.

All column cartridges were conditioned using 5 mL of acetone, 5 mL of methanol, and 10 mL of Milli-Q water. Throughout extraction, the flow rate was maintained at 10 mL/min by controlling the vacuum gauge at −15 inch Hg. The cartridges were left to dry under vacuum for about 30 minutes after all the samples had passed through. Elution
was perfumed with $2 \times 5 \text{ mL}$ of acetone. 50 $\mu$L of DMSO was added before the acetone solvent was evaporated off under a gentle stream of nitrogen. The extracts were stored at $-20^\circ \text{C}$ until E-SCREEN assays were performed.

**E-SCREEN assay**

The assay was carried out with some simplifications from the original method by Soto et al. (1995). In brief, subconfluent MCF-7 ATCC cells grown in 25 cm$^2$ flasks in growth medium were trypsinized, washed with culture medium and resuspended in steroid-free experimental medium. Cells were seeded into 24 well plates at a density of 10,000 cells per well (measured using a haemocytometer). After 24 hours, the medium was then aspirated and replaced by 1 mL of experimental medium containing one of five different solutions of an effluent extract. Each dilution was tested in quadruplicate per assay. Four assays per well without hormones were the negative control. E2 in five various concentrations between $10^{-12} \text{ M}$ and $10^{-8} \text{ M}$ served as positive control in each assay. After five days incubation, the assay was terminated during the late exponential phase of proliferation by determination of the cell number in each well. Instead of counting the cells of nuclei directly, cell numbers were assessed by measurement of total protein content using sulforhodamine B (SRB) assay. Within a wide range the extinction of SRB at 550 nm (reference 630 nm) is directly proportional to the cell number. The procedure of the SRB is described in detail elsewhere (Körner et al., 1998, 1999).

**Quantitative evaluation of the E-SCREEN assay results**

Proliferative effect (PE) can be achieved by ascertaining the endpoint of E-SCREEN assay. The endpoint of the E-SCREEN bioassay is the number of cells after treatment compared to the number of cells in a negative control sample (cell treated with estrogen-free medium supplemented with solvent only). Thus, PE is defined as the ratio of the highest cell number obtained with the tested chemical to the cell number of the hormone-free control (Soto et al., 1995).

$$\text{PE} = \frac{\text{cell number}_{\text{max}} (\text{sample})}{\text{cell number} (\text{negative control})}$$

(1)

The estrogenic activity of the test compound is evaluated by the determination of the relative proliferative effect (RPE), the relative efficacy. This compares the maximal proliferation induced by a test compound with that induced by E2. Thus, full agonists (RPE = 100%) can be distinguished from partial agonists (RPE < 100%).

$$\text{RPE} = \frac{[\text{PE (sample)} - 1]}{[\text{PE (E2)} - 1]} \times 100\%$$

(2)

The estrogenic activity of the test compound also can be evaluated by total content of estrogenic activity: the 17ß-estradiol equivalent concentration (EEQ), which is the total concentration of estrogenic active compounds in an environmental sample normalized to E2 concentration. The EEQ is calculated as follows.

$$\text{EEQ} = \frac{\text{EC}_{50} [\text{E2}]}{\text{EC}_{50} [\text{sample}]}$$

(3)

where $\text{EC}_{50} [\text{E2}]$ is the concentration of E2 at which 50% of the PE is achieved as positive control and $\text{EC}_{50} [\text{sample}]$ is the concentration factor at which 50% of the PE is achieved.

For all samples, standard curve fitting and $\text{EC}_{50}$ calculations were based on the sigmoid model (GraphPad Prism, 4.0 for Windows, GraphPad Software, San Diego, USA).
shown as follows. General statistical analysis was conducted using Microsoft Excel 2002.

\[
\text{Response} = \frac{\text{bottom} + (\text{top} - \text{bottom})}{(1 + 10^{\log \text{EC50} - x})^4}
\] (4)

Results and discussion

E-SCREEN assay simplification and validation for wastewater samples

Instead of direct counting of cells, the endpoints that are proportional to the cell number are determined by simple colorimetric assays. To validate the use of SRB assay as an endpoint, a calibration curve was obtained. Cells were seeded into a 24-well plate within the range of 0–500,000 cells per well, and the cells were allowed to attach to the bottoms of the wells. After five hours, the SRB assay was performed. A correlation between the number of cells per well and the extinction value measured at 550 nm (reference 630 nm) was obtained. An $R^2$ of 0.9981 was obtained, reflecting a good fit in this correlation, thus implying that the SRB assay is a good method to ascertain the endpoints of the E-SCREEN assay.

To validate the simplified E-SCREEN assay, industrial chemicals (Bisphenol-A, 4-tert-butylphenol, 4-chloro-3-methylphenol and 4-chloro-2-methylphenol) with known estrogenic activity in vitro and partly in vivo were tested. Each chemical was tested in at least three independent experiments, each carried out in quadruplicate. As shown in Table 1, it is clear that Bisphenol-A (BPA) and 4-tert-butylphenol (4TBP) have high RPE (more than 60%). On the other hand, 4-chloro-3-methylphenol and 4-chloro-2-methylphenol have low RPE, ranging from 30 to 40%. Thus, full agonists (RPE > 60%) can be distinguished from partial agonists (RPE < 60%). Additionally, the results obtained for BPA and 4TBP are generally in good agreement with those published by Soto et al. (1995) and Villalobos et al. (1995). Interestingly, the two phenolic compounds, 4-chloro-3-methylphenol and 4-chloro-2-methylphenol, not previously tested for estrogenic properties, expressed a weak but clear estrogenic activity. It means that simplified E-SCREEN assay is a sensitive and reliable tool, with an endpoint suitable for screening for their receptor-mediated estrogenic potency.

Estrogenic activities of effluent samples, with pH adjustment to 2.5 or 7.0, were shown in Table 2. It is apparent that at pH 7, the RPE for the effluent is smaller than the RPE obtained at pH 2.5. This is totally different for the values obtained by Körner et al. (1999), which state that the lower the pH, the lower the PE. It could be caused by the difference in SPE apparatus, or that the specific estrogenic compounds that are present in treated effluent samples in the current study were likely extracted more easily at lower pH.

Effects of chlorination conditions on estrogenic activities

In order to avoid cytotoxic effects of the DMSO, 50 µL DMSO extracts had to be diluted with experimental medium at least to 50 ml, so a maximum concentration of 0.1% DMSO was achieved for the assay. Thus, testing of extracts of 50 ml which were

Table 1 Quantitative determination of the estrogenic activity of two xenoestrogens and two phenolic compounds with E-SCREEN assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>PE</th>
<th>RPE (% E2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol (E2)</td>
<td>3.90 ± 1.11</td>
<td>100</td>
</tr>
<tr>
<td>Bisphenol-A</td>
<td>3.70 ± 0.80</td>
<td>93.27 ± 5.11</td>
</tr>
<tr>
<td>4-tert-butylphenol</td>
<td>2.81 ± 0.42</td>
<td>62.32 ± 7.75</td>
</tr>
<tr>
<td>4-chloro-3-methylphenol</td>
<td>2.00 ± 0.33</td>
<td>34.58 ± 4.08</td>
</tr>
<tr>
<td>4-chloro-2-methylphenol</td>
<td>2.17 ± 0.20</td>
<td>40.44 ± 5.25</td>
</tr>
</tbody>
</table>
extracted from 1 L samples resulted in a concentration factor of 20. In the present study, the concentration factor ranged from 0.02 to 20.

The results of the E-SCREEN tests of samples are summarized in Table 3. A clear trend was observed when the chlorine dosage was varied. The total estrogenic activity of samples ranged between 22.40 ng/L EEQ for non-chlorinated secondary effluent, and 15.02–8.35 ng/L EEQ for effluents chlorinated with 1–4 mg/L of chlorine. It indicates that chlorination could alter the estrogenic structure of endocrine disrupting compounds and thus destroyed their estrogenic activities, through strong oxidizing power of chlorine. The RPE of the secondary effluent was 35.02% (<60%), which means that it contained low amounts of full ER agonists, but full agonistic activities of samples were found after chlorination, because RPE increased to 61.60–95.53%. This may be because the chlorination destroyed more antagonistic activities of anti-estrogens at the same time when it altered the estrogenic structure of endocrine disrupting compounds, thus leading to higher full ER agonists in effluents.

There was a clear relationship between contact time and overall estrogenic activity of effluents. It was shown in Table 3 that increasing the contact time from 10 to 30 minutes produced increasing EEQ from 14.04 ng/L to 29.97 ng/L. The final estrogenic activity of a chlorinated water sample could be a combination of conflicting functions of chlorination, which are the alteration of the estrogenic structure of endocrine disrupting compounds and the production of chlorinated by-products. Therefore, the increase of overall estrogenicity of effluent with the increase of contact time may be mainly caused by the increase of CBPs concentration. The RPE of the samples were between 68.73% and 90.82%, showing an increased full agonistic activity in the chlorinated samples.

Table 3 also showed an obvious relationship between the TOC concentration and estrogenic activity of effluents. The studied samples were pure water, spiked using
commercial humic acid of various concentrations to produce the desired TOC levels. EEQ of between 19.69 and 27.20 ng/L was seen when TOC levels of 5, 10 and 15 mg/L (in the form of commercial humic acid) were used. A TOC level of 5 mg/L produced the smallest overall estrogenicity, and a TOC level of 15 mg/L produced the biggest overall estrogenicity. It can be concluded that the chlorination of humic acid does produce CBPs which are estrogenic. This concurs very well with the observations of Itoh et al. (2000) that unknown chlorinated humic acid products might exhibit estrogenic activity and that an increase in the concentration of humic acid will result in increased levels of those products and consequently increased estrogenic activities. The RPE of the samples were between 59.12% and 96.88%, showing higher full agonistic activity detected by the E-SCREEN assay when the TOC level was higher than 10 mg/L.

The relationship between estrogenicity of effluents and temperature ranging from 5°C to 20°C is not apparent (data not shown). Although temperatures have been proven to be an important factor in determining the species of chlorinated by-products (Lee et al., 2001), during the course of assay which lasted for about five days, the chemistry within the samples could have changed and thus could not present any observable difference. Similarly, no clear relationship between pH of effluents and estrogenic activity could be ascertained (data not shown). One reason for this unclear relationship is probably due to the fact that the lessening of pH from 5.5–8.5 to pH 3.0 prior to extraction in order to improve extraction efficiencies may have disturbed the matrix inside the samples where the chlorinated by-products were concerned. By-products could have changed forms due to pH change, affecting their estrogenic activities.

Conclusions
From the experiments that have been conducted, the following conclusions can be drawn. The increase of chlorine dosages, in the range 0–4 mg/L, decreased the estrogenic activity of chlorinated effluents. However, the increase of contact time, in the range from 10–30 minutes, increased the estrogenic activity of chlorinated effluents. Similarly, increasing TOC levels of between 5 mg/L and 15 mg/L in the form of commercial humic acid resulted in an increase of estrogenic activity in water. However, clear relationships between pH and temperature and estrogenic activity of chlorinated effluents could not be observed.

References
