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ARTICLE in CHEMOSPHERE · OCTOBER 2013
Impact Factor: 3.34 · DOI: 10.1016/j.chemosphere.2013.09.073 · Source: PubMed

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Removal potential of anti-estrogenic activity in secondary effluents by coagulation

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Highlights
- DOM fractions <3000 Da in HOA and HON of secondary effluents were two key anti-estrogenic fractions.
- The anti-estrogenic activity of secondary effluents was limitedly removed by coagulation.
- The two key anti-estrogenic fractions were refractory during coagulation.

Abstract
Anti-estrogenic activity in wastewater is gaining increased attention because of its endocrine-disrupting function. In this study, the level and removal efficiency by coagulation of anti-estrogenic activity in secondary effluents of domestic wastewater treatment plants were studied. Anti-estrogenic activity was detected in secondary effluent samples at a tamoxifen (TAM) equivalent concentration level of 0.38–0.94 mg-TAM L⁻¹. Dissolved organic matters (DOM) with the molecular weight (MW) less than 3000 Da in hydrophobic acids (HOA) and hydrophobic neutrals (HON) fractions of the secondary effluent were the key fractions related to anti-estrogenic activity. Coagulation with FeCl₃ and polyaluminium chloride (PAC) can remove the anti-estrogenic activity of the secondary effluents, but the removal efficiency was limited. The removal efficiency using FeCl₃ coagulant was higher than that induced by PAC. Dissolved organic carbon was continuously removed with increased coagulant dose (0–120 mg L⁻¹ FeCl₃ or 0–60 mg L⁻¹ PAC). However, the removal of anti-estrogenic activity was not enhanced further when the coagulant concentration was beyond a critical value (30 mg L⁻¹ FeCl₃ or 10 mg L⁻¹ PAC). The highest removal of anti-estrogenic activity was about 36% by FeCl₃ and 20% by PAC. Size exclusion chromatography results indicated difficulty in removing DOM with MW less than 3000 Da in the secondary effluent during coagulation even at a high coagulant concentration, which led to low removal efficiency of anti-estrogenic activity.

1. Introduction

Wastewater reuse is an important method of dealing with the global water crisis. Nevertheless, conventional treatment processes in wastewater treatment plants (WWTPs) fail to remove all synthesized and natural organic chemicals in effluents. Endocrine-disrupting chemicals (EDCs) are widely detected in WWTP effluents.
EDCs are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural bodily hormones that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (USEPA, 1997). EDCs are also considered to be potentially harmful to wildlife (Colborn et al., 1993).

Today, anti-estrogenic activity, a typical endocrine-disrupting effect, is frequently detected in surface water, municipal, and industrial wastewater. Improper contact and intake of chemicals or medicines with anti-estrogenic activity reportedly disrupt the function of the endocrine system of organisms, leading to masculine characteristics in females (Hodgson, 2004). Therefore, the presence of anti-estrogenic compounds in the environment adds to the potential ecological risk.

Since the 1990s, chemicals with anti-estrogenic activity have been reported in surface water and wastewater. Some natural phytochemicals and wood extracts found in pulp mill effluent exhibit anti-estrogenic activity (Collins et al., 1997; Orrego et al., 2010). Synthetic chemicals and treatment by-products from industrial and municipal wastewater, such as some polynuclear aromatic hydrocarbons (Tran et al., 1996) and some plasticizers (Okubo et al., 2003), also show anti-estrogenic activity and are discharged into aquatic environments. In research report, surface water has been affected by secondary effluents with anti-estrogenic activity (Vega-López et al., 2007).

Researchers are also attempting to monitor and control anti-estrogenic activity in wastewater and reclaimed water. Conroy et al. (2007) found that after storage, the anti-estrogenic activity dramatically increases. Moreover, Stalter et al. (2011) concluded that anti-estrogenic activity is undetectable in the influent but appears in the effluent of a secondary clarifier possibly because of the effective removal of estrogen agonists during conventional activated sludge treatment.

In recent years, researchers have also begun to evaluate the effects of tertiary treatment on anti-estrogenic activity in reclaimed water. Wu et al. (2009) found that chlorination increases the anti-estrogenic activity of secondary effluents. Stalter et al. (2011) found that ozonation and activated carbon do not decrease the anti-estrogenic activity of secondary effluents, but even increase the anti-estrogenic activity after ozonation.

In wastewater reclamation plants, coagulation combined with filtration is widely used as an effective method of removing organic colloids. Coagulants such as FeCl₃, PAC, polyalumminium silicate sulfate, and aluminum sulfate demonstrate high efficiency in removing SS and turbidity in reclaimed water, which helps reduce membrane fouling for the post-filtration process (Abdessemed et al., 2000; Haberkamp et al., 2007; Baek and Chang, 2009). FeCl₃ can also reportedly form stronger and heavier flocs over a broader pH range and is less sensitive than alum in case of poor filtrate quality caused by overdosing (Stephenson and Duff, 1996). Arnaldos and Pagilla (2010) suggested that alum can remove dissolved organic nitrogen and dissolved non-reactive phosphorus.

However, the effects of coagulation on the anti-estrogenic activity in secondary effluents or reclaimed water are still hardly known.

This study aimed to assess the effects of coagulation on anti-estrogenic activity in secondary effluents, and identify the key factors related to anti-estrogenic activity in secondary effluents.

2. Materials and methods

2.1. Sampling

Secondary effluent samples were collected from two municipal WWTPs (WWTP-Q and WWTP-G) in Beijing, China. The anaerobic–anoxic–oxic process is used in WWTP-Q, and the oxic–anoxic–anaerobic process is used in WWTP-G. Samples were kept at 3–6 ºC on ice, and immediately transported to the laboratory for water quality measurement, preparation, and coagulation experiments within 24 h.

2.2. Water quality analysis

Water samples before and after coagulation were passed through 0.45 μm glass filters before water quality analysis. The pH was measured with a Mettler Toledo Fe20 analyzer. The concentration of dissolved organic carbon (DOC) in water samples was measured with a Shimadzu TOC-5000A analyzer. UV absorbance was measured with a Shimadzu UV-2401PC UV–VIS recording spectrophotometer. Measurements were performed in triplicate.

2.3. Coagulation experiments

Coagulation experiments were performed on a coagulation instrument in laboratory. One liter of secondary effluent sample was used for coagulation, and the coagulants FeCl₃ and PAC were used at the dosages of 0–120 mg L⁻¹ for FeCl₃ and 0–60 mg L⁻¹ for PAC. In addition, the maximum coagulant concentrations were beyond the actual condition by about 100%, so as to obtain convincing and significant results.

Coagulants were added to the sample after 30 s of rapid stirring (200 r min⁻¹). Stirring (150 r min⁻¹) was continued for 2 min, and the samples were left undisturbed for 30 min to enable precipitation. The pH monitoring during coagulation revealed that it was about 7.6 before coagulation and insignificantly varied thereafter.

2.4. Fractionation by resin adsorption

According to the original protocol of Leenheer (1981) and a modified one introduced by Zhang et al. (2009), nonionic macroporous resin Supelite XAD-8 (20–60 mesh), cation-exchange resin Dowex Marathon MSC (H⁺) (20–50 mesh), and anion-exchange resin Duolite A-7 (free base) were selected, purified and conditioned. Then they were respectively packed into glass columns (0.25 L) for fractionation. After preparing the resins, 1 L of water sample filtrate was fractionated into six fractions as in the procedure shown in Supplemental Fig. 1 (Zhang et al., 2009). Thereafter, all six fractions were diluted to the original sample volume (1 L) with ultrapure water.

2.5. Size exclusion chromatography

Size exclusion chromatography (SEC) was used for the molecular weight (MW) analysis of the fractions on a Shimadzu LC-20 high-performance liquid chromatography system combined with a Shimadzu SPD-M20A UV detector and two connected columns (a TSK-GEL G3000PWXL column followed by a TSK-GEL G2500PWXL column).

The columns were kept at 40 ºC, and the mobile phase was composed of MILLI-Q ultrapure water buffered with phosphate (0.0024 M Na₂HPO₄ and 0.0016 M Na₂HPO₄) and 0.025 M sodium sulfate. For each measurement, 100 μL of the fractions was injected and UVA signals were monitored during the following 60 min. The MW standards used were polyethylene glycol (330, 700, 1050, 5250, 10225, and 30000 Da) and acetone. After calibration, the retention time in the SEC chromatogram was converted to the MW, and the baseline was adjusted to a zero line.
2.6. Fractionation by ultrafiltration after resin adsorption

The HOA and HON fractions (500 ml) were further fractionated using a Millipore Amicon Model-8200 membrane ultrafiltration system with Millipore 3000 Da cellulose filtration membranes. By ultrafiltration, two sub-fractions with MW more than 3000 Da and less than 3000 Da were each obtained for HOA and HON. Thereafter, both sub-fractions were diluted to the original volume (500 ml) for further analysis and bio-assays.

2.7. Sample concentration

Water samples (250 ml) before and after coagulation were acidified to pH 2 with 2 M H2SO4, and then passed through Waters Oasis HLB resin cartridges. After adsorption, the cartridges were dried under a flow of air. Adsorbed organics on the cartridge were eluted with methanol (10 ml), followed by dichloromethane (10 ml) and hexane (10 ml) to obtain a mixture of sample extracts. Thereafter, the mixture was completely dried under nitrogen flow. The dry residues were dissolved in 125 μl of dimethylsulfoxide to obtain a 2000-fold concentration (volume of sample/volume of extract) for anti-estrogenic activity assay.

2.8. Estrogenic activity assay

The estrogenic activity of the samples was evaluated with the yeast two-hybrid assay based on yeast cells (Saccharomyces cerevisiae Y190), and the β-galactosidase induced by estrogenic chemicals was used to monitor the estrogenic activity (Nishikawa et al., 1999). The assay was conducted as in the detailed protocol described by Wu et al. (2009), and absorbance at 415, 570, and 595 nm was converted to ethanol units according to the equation described by Nishikawa et al. (1999). After obtaining the dose–response curve of standard 17β-estradiol (E2) and concentrated samples, the estrogenic activity values of the samples were given as an E2-equivalent (Wu et al., 2009).

2.9. Anti-estrogenic activity assay

The ability of the concentrated sample to inhibit the β-galactosidase activity of E2 was measured to determine the anti-estrogenic activity of the concentrated sample according to the yeast two-hybrid assay (Shiraishi et al., 2001; Jung et al., 2004).

According to the procedure introduced by Wu et al. (2009), an anti-estrogenic standard chemical tamoxifen (TAM) was used. The inhibitions of the concentrated sample and TAM to β-galactosidase induced by E2 were measured, and the dose–response curves of the sample and standard TAM were generated. Afterwards, the anti-estrogenic activity of the sample was given as a TAM equivalent.

2.10. Data processing and statistics

Experimental data were processed and figures were performed with the software Origin version 8. Particularly, for the SEC data, the peak area (X-axis, molecular weight; Y-axis, UV absorbance) in each SEC curve was calculated.

Besides, when the estrogenic or anti-estrogenic activity data for samples with different coagulant concentrations were obtained after the triplicate measurements, t-tests was employed to see if there was significant difference between them with the different coagulant concentrations.

3. Results and discussion

3.1. Key fractions with anti-estrogenic activity in secondary effluents

The water quality and anti-estrogenic activity of each sample are shown in Table 1. None of the three samples exhibited estrogenic activity, but all showed high level of anti-estrogenic activity. According to the reports of Vega-López et al. (2007), Conroy et al. (2007) and Wu et al. (2009), secondary effluents always exhibit anti-estrogenic activity, especially when there is low level of estrogenic activity.

Using the fractionation method described in Section 2.4, each sample was fractionated into six fractions, and the anti-estrogenic activity of those fractions was measured (Fig. 1). HOA and HON were the key fractions with high levels of anti-estrogenic activity, and HIN was also observed to be anti-estrogenic in samples A and C at low levels. HOB, HIA, and HIB exhibited no anti-estrogenic activity. These results agreed with the report of Conroy et al. (2005) that hydrophobic organics in secondary effluent were the key fraction related to anti-estrogenic activity.

Given that HOA and HON showed high levels of anti-estrogenic activity, focus should be directed onto their removal efficiencies during wastewater reclamation.

3.2. Removal of anti-estrogenic activity in secondary effluents during coagulation

To examine the removal of anti-estrogenic activity in secondary effluents during coagulation, sample B was further used in coagulation experiments in our laboratory. The results of DOC and anti-estrogenic activity changes during coagulation are shown in Fig. 2.

Unlike that ozonation, as tertiary treatment, led to increase of anti-estrogenic activity in secondary effluents (Stalter et al., 2011), coagulation did exert positive effects on the removal of anti-estrogenic activity.

DOC decreased to 9.1 mg L\(^{-1}\) with FeCl\(_3\) and 12.1 mg L\(^{-1}\) with PAC at the highest doses (120 mg L\(^{-1}\) FeCl\(_3\) or 60 mg L\(^{-1}\) PAC) of this study, reaching removal efficiencies of 38% and 18%, respectively. The increase in DOC elimination with increased coagulant doses indicated that co-precipitation by metal hydroxide was the predominant mechanism for DOM removal. Similar correlation between DOC and coagulant was also supported by Yan et al. (2006) and Haberkamp et al. (2007). Moreover, FeCl\(_3\) exhibited a higher DOC removal efficiency than PAC.

On the other hand, anti-estrogenic activity decreased and the highest removal percentages were about 36% (by FeCl\(_3\)) and 20% (by PAC). However, unlike the DOC removal, with increased coagulant dose, the anti-estrogenic activity was quickly eliminated at low doses (0–30 mg L\(^{-1}\) FeCl\(_3\) or 0–10 mg L\(^{-1}\) PAC) but insignificantly changed after reaching a critical dose (30 mg L\(^{-1}\) FeCl\(_3\) or 10 mg L\(^{-1}\) PAC). DOC was continuously eliminated with increased coagulant dose, which indicated that the anti-estrogenic constituents of residual DOM difficulty were difficult to remove during coagulation. According to the mechanism of coagulation (Bratby, 2006) and the conditions of coagulation experiments (a normally applied coagulation operating condition), hypotheses can be proposed that some refractory hydrophobic anti-estrogenic constituents may be hard to be co-precipitated with the metal hydroxide; or they are easily re-stabilized during hydraulic mixing, etc. However, these hypotheses need to be verified in further research.

Therefore, increasing only the coagulant dose is ineffective for enhancing the removal of anti-estrogenic activity, and residual DOM after coagulation requires further study.
3.3. Removal of anti-estrogenic activity in fractions during coagulation with FeCl₃

As aforementioned, FeCl₃ demonstrated higher efficiencies in removing DOC and anti-estrogenic activity than PAC; hence sample B during coagulation with FeCl₃ was further studied.

The critical dose observed in the removal curve for anti-estrogenic activity by FeCl₃ was 30 mg L⁻¹. To identify the key constituents limiting the removal of anti-estrogenic activity, samples before and after coagulation (30 and 120 mg L⁻¹) by FeCl₃ were fractionated. Changes in DOC (Supplemental Fig. 2) and anti-estrogenic activity (Fig. 3) of the fractions were monitored.

Supplemental Fig. 2 shows the differences in DOC removal among the six fractions. HOA and HON were significantly removed because of their hydrophobicity, and the removal efficiencies increased with increased FeCl₃ dose. HIA removal also increased with the increased coagulant dose.

The changes in anti-estrogenic activity of each fraction are shown in Fig. 3. As mentioned in Section 3.1, only the HOA and HON fractions (for sample B) were detected to be anti-estrogenic (0.52 mg-TAM L⁻¹ for HOA and 0.75 mg-TAM L⁻¹ for HON) before coagulation. At the FeCl₃ dose of 30 mg L⁻¹ (the critical dose), the anti-estrogenic activity of HOA and HON decreased to 0.39 and 0.60 mg-TAM L⁻¹, corresponding to 25% and 20% removals, respectively. With increased dose of FeCl₃ to 120 mg L⁻¹, the anti-estrogenic activity remaining in HOA and HON were 0.38 and 0.52 mg-TAM L⁻¹, and the extra removal rates were only 2% and 11%, respectively. Thus, the anti-estrogenic activity of HOA and HON were unable to be further significantly eliminated with increased FeCl₃ dose after the critical dose. This phenomenon was consistent with the appearance of the critical dose during the removal of anti-estrogenic activity of secondary effluents.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>DOC (mg L⁻¹)</th>
<th>UV₂₅₄ (cm⁻¹)</th>
<th>pH</th>
<th>Anti-estrogenic activity (mg-TAM L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.6 ± 0.5</td>
<td>0.11 ± 0.01</td>
<td>7.2 ± 0.1</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td>B</td>
<td>13.7 ± 0.8</td>
<td>0.13 ± 0.01</td>
<td>7.6 ± 0.1</td>
<td>0.88 ± 0.02</td>
</tr>
<tr>
<td>C</td>
<td>10.6 ± 0.5</td>
<td>0.10 ± 0.01</td>
<td>7.3 ± 0.1</td>
<td>0.38 ± 0.04</td>
</tr>
</tbody>
</table>

*Values are given as the mean value ± standard deviation after triplicate measurements.*

---

**Fig. 1.** Anti-estrogenic activity of fractions in secondary effluents. HOA, HOB and HON are hydrophobic acids, bases and neutrals, and HIA, HIB and HIN are hydrophilic acids, bases and neutrals, respectively. Error bars represent the standard deviation based on triplicate measurements. "--" sign means the value is below the limit of detection.

**Fig. 2.** Removal of anti-estrogenic activity and DOC in secondary effluents during coagulation. Two types of coagulants FeCl₃ and polyaluminium chloride (PAC) are used for coagulation. Error bars represent the standard deviation based on triplicate measurements.

**Fig. 3.** Changes in anti-estrogenic activity of fractions during coagulation with FeCl₃. HOA, HOB and HON are hydrophobic acids, bases and neutrals, and HIA, HIB and HIN are hydrophilic acids, bases and neutrals, respectively. Error bars represent the standard deviation based on triplicate measurements. "--" sign means the value is below the limit of detection.
3.4. Changes in MW distribution during coagulation with FeCl₃

The anti-estrogenic activity of the secondary effluent sample and its fractions remained at a relatively high level after the critical coagulant dose. Thus, residual DOM needed to be characterized to find out the refractory anti-estrogenic constituents.

Coagulation is known to be effective in removing colloid and DOM with large MW. The changes in MW distribution of DOM in sample B and its fractions were monitored to determine any correlation between the MW distribution changes and anti-estrogenic activity.

SEC with a UV detector at 254 nm was used to characterize the MW distribution, and Supplemental Fig. 3 presents the changes in MW distribution of DOM in sample B during coagulation. UV signals were mainly observed within the retention time of 28–36 min, which covered the MW range of 1000–20000 Da. At the FeCl₃ concentration of 120 mg L⁻¹, DOM with MW of 3000–20000 Da was remarkably removed (corresponding peak area decreased by 62%), and DOM with MW more than 10000 Da was nearly eliminated completely. However, DOM with MW 1000–3000 Da was eliminated limitedly (corresponding peak area decreased by 27%) at the same FeCl₃ concentration. Both Haberkamp et al. (2007) and Wert et al. (2011) reported that with increased dose of the coagulant FeCl₃, biopolymers and high-MW humic substances were efficiently removed; the concentration of low-MW neutrals were minimally removed at all coagulant dosages. In addition, humic substances, aggregations of mixtures and always with molecular weight around 10 k Da or even more than 100 k Da (Simpson et al., 2002), were determined to be anti-estrogenic (Jahnosek et al., 2007; Wu et al., 2009). Therefore, it was suggested that coagulation may remove quantity of humic substances, which resulted in a decrease of the anti-estrogenic activity.

The SEC chromatograms of HOA and HON during coagulation are shown in Fig. 4. For HOA, DOM was minimally removed at the FeCl₃ concentration of 30 mg L⁻¹, especially for DOM with MW more than 3000 Da. When the FeCl₃ concentration was increased to 120 mg L⁻¹, DOM with MW of 3000–20000 Da was significantly removed (corresponding peak area decreased by 57%), and the removal of DOM with MW more than 10000 was almost 100%. However, at the FeCl₃ concentration of 120 mg L⁻¹, the DOM with MW of 1000–3000 Da was eliminated only a little (corresponding peak area decreased by 18%). For HONs, nearly the same patterns of DOM removal were observed, except for better removal for each MW interval at 30 mg L⁻¹ FeCl₃.

Compared with the removal of both anti-estrogenic activity and DOM with MW less than 3000 in HOA and HON, resistance to increased coagulant dose after the critical dose was observed. The similarities between the removal of anti-estrogenic activity and DOM with less than 3000 Da in HOA and HON indicated that DOM less than 3000 Da in HOA and HON may account for the majority of anti-estrogenic activity.

Sub-fractions with MW more than 3000 Da and MW less than 3000 Da for HOA and HON respectively were prepared after fractionation by ultrafiltration, and their anti-estrogenic activity was measured (Fig. 5). The results revealed that sub-fractions with MW less than 3000 Da in HOA and HON contributed the most to anti-estrogenic activity in the sample, and these sub-fractions were suggested to result in the high level of residual anti-estrogenic activity after coagulation.

What's more, Shon et al. (2006) noted that DOM less than 10¹³ Da in waste/secondary effluents often contained carbohydrates, amino acids, and some artificial chemicals such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls, etc. Further, DOM with small MW was also reported to be related to bio-toxicity. For example, reported typical EDCs are almost with small MW, and they are harmful constituents in DOM (Westerhoff et al., 2005). Wu et al. (2010a) found that DOM with MW less than 1000 Da is highly related to genotoxicity in secondary effluents. Moreover, in a relevant study focusing on anti-estrogenic activity in secondary effluents, an anti-estrogenic by-product generated...
from amino acids during chlorination was identified (Wu et al., 2010b). Therefore, the DOM fractions with MW less than 3000 Da or even smaller in secondary effluents should be paid more attention to, for it may be of high bio-toxic and refractory during tertiary treatment, such as conventional coagulation.

4. Conclusions

Secondary effluent samples showed anti-estrogenic activity (0.38–0.94 mg-TAM L$^{-1}$), and fractions with MW less than 3000 Da in HOA and HON were the key fractions with high levels of anti-estrogenic activity in the secondary effluents investigated in this study.

Coagulation with FeCl$_3$ and PAC decreased the anti-estrogenic activity in secondary effluents. However, with increased coagulant doses, anti-estrogenic activity removal reached maximum values at a critical coagulant dose (30 mg L$^{-1}$ FeCl$_3$ or 10 mg L$^{-1}$ PAC), beyond which no further removal was observed. Both HOA and HON were resistant to the increasing coagulant dose after the critical dose. SEC analysis showed that DOM with MW less than 3000 Da was inefficiently eliminated during coagulation, leading to low removal of anti-estrogenic activity in the secondary effluents.

All these results suggested that coagulation decreased the anti-estrogenic activity of secondary effluents, but those constituents with MW less than 3000 Da and high-level anti-estrogenic activity may still remain in the effluents after coagulation.

Acknowledgements

This study was funded by National Science Fund of China (Nos. 51138006, 51208278) and National High-tech R&D Program of China (863 Program) (No. SS2013AA061805).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.09.073.

References