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Characteristics of estrogenic/antiestrogenic activities during anoxic/aerobic biotreatment process of simulated textile dyeing wastewater

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Abstract

The presence of estrogenic/antiestrogenic chemicals in textile dyeing wastewater is well demonstrated according to previous studies. However, the characteristics of estrogenic/antiestrogenic activities during the conventional biological treatment has been poorly investigated. In this study, the yeast two-hybrid assay (YES) was used to evaluate the agonistic and antagonistic estrogen activity during the anoxic/aerobic treatment of textile dyeing wastewater. The results indicated that the estrogenic activity of the textile dyeing wastewater was negligible throughout the anoxic/aerobic treatment, but the antiestrogenic activity increased obviously after the aerobic treatment. By fractionating the dissolved organic matter (DOM) in wastewater into different fractions, it was found that hydrophobic acids (HOA) and hydrophobic neutrals (HON) were the key fractions involved in increasing antiestrogenic activity of the wastewater during anoxic/aerobic treatment. Furthermore, fluorescence spectroscopy analysis on wastewater samples and their fractions of soluble organic compounds suggested that HOA and HON fractions contained more humic/fulvic acid in aerobic effluent than that in anoxic effluent, which could mask estrogenic activity in aerobic effluent.

Keywords: estrogenic/antiestrogenic activity; simulated textile dyeing wastewater; anoxic/aerobic treatment; yeast two-hybrid assay (YES); DOM fractionation

1. Introduction

Biological techniques are ubiquitously used in textile dyeing wastewater treatment in wastewater treatment plants (WWTPs) for their high-efficiency and low-cost [1]. Although biological treatment successfully removes the great mass of organic matters resulting in compliance with discharge standard, some trace organic matters like estrogenic/antiestrogenic chemicals are biodegraded incompletely [2, 3]. The estrogenic/antiestrogenic chemicals have been reported to mimic or antagonize the effect of steroid hormones, and interfere with the function of the endocrine system through affecting the reproduction and development of animals [4-6]. Recently, it has been demonstrated that there are estrogenic/antiestrogenic activities in textile dyeing effluent. Therefore, much more efforts should be made to investigate the fate of estrogenic/antiestrogenic chemicals in textile dyeing wastewater treatment [7, 8].

Textile dyeing wastewater usually contains variety of dyes and the auxiliaries released from textile dyeing and printing process [9-11], which act as endocrine-disrupting compounds (EDCs). A study on 23 commercial textile dyes suggested that over 50% dyes had antiestrogenic effect, and about 13% were estrogenic [12]. Some dyes can affect the endocrine function at genetic level. For example, Disperse Yellow 7 and Bismarck Brown Y were able to alter the expression of reproductive-related genes in western clawed frog [13, 14]. There are also some textile auxiliaries with estrogenic/antiestrogenic activities such as nonylphenols, which are included in the list of priority substances in the field of water policy established by the European Parliament [15].

Because not all the estrogenic/antiestrogenic chemicals can be removed effectively by conventional process [16, 17], the advanced treatment process was implemented for tertiary stage such as ozonation, but costly and not always applicable [18-20]. In the ultimate treatment for wastewater reclamation, some

estrogenic/antiestrogenic chemicals generates during the wastewater disinfection such as chlorination [21]. Up to now, study on the fate of estrogenic/antiestrogenic chemicals during biological treatment is highly concerned and conducive to better treatment of textile dyeing wastewater [22-24]. Notablely, dissolved organic matters (DOM) in the textile effluent, containing soluble microbial products and unknown estrogenic/antiestrogenic chemicals, is changeable along with biological degradation and synthesis [25, 26]. Therefore, DOM plays an important role in better evaluation of the estrogenic/antiestrogenic activities of the textile dyeing wastewater.

Accordingly, the purpose of this paper was to investigate the change characteristics of estrogenic/antiestrogenic activities during anoxic/aerobic biotreatment of textile dyeing wastewater, and further analyze the constituent and degradation products in the wastewater, which might be related to the estrogenic/antiestrogenic activities.

2. Materials and Methods

2.1 Lab-scale reactor system and simulation of textile dyeing wastewater

A sequential anoxic-aerobic reactor system was built for simulating the anoxic/aerobic treatment process of textile dyeing wastewater. The effective volume of anoxic reactor and aerobic reactor were 16 L and 9.6 L, respectively. Each reactor was followed by a sedimentation basin of effective volume 8 L. The initial seed sludge was collected from the returning activated sludge of secondary sedimentation tank in the sewage treatment plant in Songjiang, Shanghai, China. Firstly, the initial sludge was aerated for 24 h. Then, the sludge was mixed with water with a proportion of 3:2 (V: V) in both the anoxic and aerobic reactor. The flow rate of influent was 20 L/d, giving a hydraulic retention times of 12.8 h and 7.68 h for anoxic and aerobic reactor, respectively. Before performing the experiment in this study, the

reactor has run for 6 months to achieve stability. During the start-up period of the system, the dissolved oxygen (DO) of anoxic reactor fluctuated from 0.20 mg/L to 0.50 mg/L, and the DO of aerobic reactor was between 3.00 mg/L to 5.00 mg/L. Besides, the solids retention times of anoxic and aerobic reactor were 15 d and 3 d, respectively.

The composition of simulated textile dyeing wastewater was shown in Supplementary Table 1. The mixture of 5 frequently-used textile dyes was 10 mg/L of Direct Red 28, Direct Yellow 12, Reactive Black 5, Reactive blue 21 and Reactive Blue 19, respectively, and the total concentration of dyes was 50 mg/L. The starch and inorganic salts were used for providing energy, carbon source, nitrogen source and other mineral substances.

2.2 Sample collection and water quality analysis

Total operation time of the reactor system was over 180 days to reach steady-state conditions, which was defined by the pH, COD_{Cr} within 5% variation in a week. The fresh prepared simulated wastewater was used as sample influent (named IN). The anoxic effluent (named AN) and aerobic effluent (named AE) samples were collected from the sedimentation basins. All the three samples were filtered through 0.45 μm microfiltration membrane to remove the insoluble substances, and then stored in 4 □. Characteristics of these three samples were listed in Table 1, including pH value, COD_{Cr}, BOD_5, total organic carbon (TOC) and decolorization ratio. The pH values were measured immediately after collection. COD_{Cr} was analyzed by microwave digestion method after centrifugation. BOD_5 was measured using 880 digital BOD test apparatus (Jiangfen, China). The TOC was measured with V-CPH TOC analyzer (Shimadzu, Japan) after filtration. The UV-Visible spectra were detected by a P-300 nanophotometer (Implen, Germany) after filtration. Decolorization of the simulated dyeing wastewater was analyzed using ADMI (American Dye Manufacturing Institute) color values method [27].

Table 1. Characteristics of three samples used in this study

Sample	pH	COD_{Cr} (mg/L)	BOD_5 (mg/L)	TOC (mg/L)	Decolorization ratio (%)
IN	7.01	985	487	367.3	-
AN	4.73	442	340	150.6	67%
AE	7.31	162	94	65.3	75%

2.3 Fractionation

DOM of the anoxic and aerobic effluent samples was isolated into hydrophobic acids (HOA), hydrophobic bases (HOB), hydrophobic neutrals (HON) and hydrophilic substances (HIS) with method described by Huang and Yeh [28], which was performed with some modification as follows: (1) 500 mL original effluent was directly passed through the XAD-8 resin column to adsorb hydrophobic bases (HOB) and hydrophobic neutrals (HON). (2) The column was back-flushed with 200 mL 0.1 M HCl to obtain HOB fraction and subsequently 100 mL ultrapure water for flushing the residual acid and HOB. (3) The effluent from Step 1 was adjusted to pH 2 and passed through column again to absorb the hydrophobic acid (HOA). The effluent of Step 3 was hydrophilic substances (HIS). (4) The column was back-flushed again with 200 mL 0.1 M NaOH and 100 mL ultrapure water for HOA fraction. (5) The HON fraction adsorbed XAD-8 resin was eluted with methanol in Soxhlet extractor. After fractionation, The HON fraction in methanol eluate was dried by vacuum-rotary evaporation. The other fractions were added with ultrapure water to increase volume to 500 mL.

2.4 Solid-phase extraction and sample concentration

The dried residues of HONs were redissolved in 2 mL methanol. 1 mL methanol solution of HON was dried under nitrogen stream and dissolved in 250 μL DMSO to obtain 1000 fold concentration for yeast screen assay. 0.2 mL HON methanol solution was also dried and dissolved in 50 mL ultrapure water for fluorescence spectroscopy analysis.

Wastewater samples IN, AN and AE, and their fractions except for HON were solid-extracted according the method reported by Wu, *et al.* [21]. In brief, 300 mL effluent samples were acidified to pH 2 with 2 M H_2SO_4 and passed through HLB cartridges (Waters Oasis, America). The organic matters retained on the cartridge were eluted by 10 mL methanol, 10 mL dichloromethane and 10 mL hexane. Then 5 mL of each eluate was mixed and dried under the nitrogen stream. The dried residues were dissolved in 150 μL dimethylsulfoxide (DMSO) to obtain 1000-fold concentration for yeast screen assay. The rest 5 mL of each eluate was also mixed and dried for GC-MS analysis. The estrogenic/antiestrogenic activities of the concentrated samples were evaluated with the yeast two-hybrid assay based on yeast cells.

2.5 Estrogenic activity assay

The recombinant yeast cells (*Saccharomyces cerevisiae* Y190) for yeast screen assay was donated from State Key Joint Laboratory of Environmental Simulation and Pollution Control (Department of Environmental Science and Engineering, Tsinghua University, PRChina), which contained rat estrogen receptor ERα and the coactivator TIF2. Estrogenic activity was evaluated by the *β*-galactosidase induced by estrogenic samples [21, 29], which performed as follows: The yeast cells were preincubated overnight at 30□. Then 100 μL overnight cells and 20 μL DMSO solution containing the samples were added into the mixture of 400 μL SD medium and incubated at 30□ for 4 h. After incubation, 150 μL yeast culture was used for absorbance at 595 nm. The residual yeast in mixed solution (370 μL) were collected by centrifugation and resuspended in 200 μL Z-buff containing 1 g/L Zymolyase 20T for digestion (15 min, 37℃). The enzymatic reaction was started by addition of 40 μL 4 g/L 2-nitrophenyl-*β*-D-galactosidase (ONPG) at 30□. After 30 min, the reaction was stopped by addition of 100 μL of 1M Na_2CO_3. Thereafter the solution was centrifuged and 150 μL supernatant was taken for

absorbance at 405 nm and 570 nm, respectively.

Some samples with strong absorbance at 595 nm could interfere experimental results, thus the observation absorbance at 595 nm need to be corrected. The blank samples in YES assay were composed of 20 μL DMSO solution of samples and 500 μL ultrapure water. Therefore, the corrected OD_{595} was calculated in terms of Lambert-Beer's law as following equation (1):

$$OD_{595COR} = OD_{595OBS} - OD_{595BLA} + OD_{595BAC} \quad (1)$$

where OD_{595COR} represents the corrected absorbance of samples at 595 nm; OD_{595OBS} is the observed absorbance of samples after 4 h incubation; OD_{595BLA} is the absorbance of the blank samples; OD_{595BAC} is the background absorbance of empty test plate.

Therefore, the *β*-galactosidase activity was calculated according to equation (2):

$$U = \frac{1000 \cdot (OD_{405} - 1.75 \cdot OD_{570})}{t \cdot v \cdot OD_{595COR}} \quad (2)$$

where U represents the *β*-galactosidase activity; the OD_{405} is the absorbance of *o*-nitrophenol after reaction; OD_{570} is light scatting after reaction, *t* is time of reaction (min); *v* is volume of culture (mL).

2.6 Antiestrogenic activity assay

The antiestrogenic activity assay was investigated by the inhibitory effect of samples against *β*-galactosidase activity of E2, also according to the yeast two-hybrid assay [30]. In this assay, 100 μL yeast culture, 20 μL DMSO solution containing the concentrated sample and additional E2 was also added to 400 μL SD medium. The final concentration of E2 was 0.77 μg/L, which could elicit 40% submaximal ER agonist response in the absence of antiestrogenic chemicals. For the correction of absorbance at 595 nm, the blank samples consisted of 10 μL DMSO solution of samples, 10 μL DMSO and 500 μL ultrapure water. After 4 h incubation, the *β*-galactosidase activity was determined, the

173 percentage inhibition of concentrated samples to the *β*-galactosidase reduction was calculated
174 according to the following equation (3):

$$I_X(\%) = \frac{U_{E2} - U_X}{U_{E2}} \cdot 100\% \quad (3)$$

175 Where I_X represents the inhibition of concentrated samples to *β*-galactosidase activity induced by E2;
176 U_{E2} is the *β*-galactosidase activity by 0.77 μg/L E2 standard; U_X is the β-galactosidase activity of E2
177 and the concentrated sample.

178 **2.7 Cytotoxicity assay**

179 Toxic samples can inhibit the growth of yeast cells, which also lead to the inhibition of
180 *β*-galactosidase activity. Therefore, the toxicity of the sample was measured by the absorbance at 595
181 nm (OD_{595}) after 4 h incubation of yeast culture during the antiestrogenic assay [21, 30]. The OD_{595} value
182 was also corrected as mentioned in estrogenic assay and then converted to percentage inhibition of the
183 concentrated sample to yeast growth, as following equation (4):

$$I_c(\%) = \frac{OD_{595b} - OD_{595x}}{OD_{595b}} \times 100\% \quad (4)$$

184 Where the I_c is the inhibition of the sample to growth of yeast cell; OD_{595b} is the absorbance after
185 incubation with E2 and DMSO; OD_{595x} is the absorbance after incubation with E2 and DMSO solution
186 containing the concentrated sample. The sample was assessed as toxic when the I_c was 10% or more
187 than 10%.

188 **2.8 Fluorescence spectroscopy**

189 Fluorescence spectral measurement was conducted using the QuantMaster 40 fluorescence
190 spectrometer (PTI, America). The fractions of sample AN and AE were adjusted to pH 3 before
191 measurement. The excitation wavelength range was 200-400 nm, at intervals of 5 nm; the emission

192 wavelength range was 280-550 nm, at intervals of 1 nm. To limit its second-order Raleigh scattering,
193 the starting wavelength of emission was 20 nm longer than its corresponding excitation wavelength
194 from beginning to end. After inner-filter correction [31], data of fluorescence spectra were converted into
195 the excitation-emission matrixes (EEM). The contour maps were created using Origin 8.0 program with
196 same scale range of fluorescence intensities.

197 **2.9 GC-MS analysis**

198 The degradation products during anoxic/aerobic biotreatment process was conducted using QP-2010
199 gas chromatography coupled with mass spectrometer (Shimadzu, Japan). The GC-MS analysis was
200 performed at ionization voltage 70 eV. The initial Restek column (0.25 mm, 60 m; XTI-5) temperature
201 remained at 40□ for 10 min, then ascend to 280℃ at 10℃ min^{-1} , and remained for 7 min. The
202 temperature of injection port kept at 280℃ and the GC/MS interface maintained at 300℃. The flow
203 rate of carrier gas (nitrogen) was 1.0 mL min^{-1}. The products were identified based on the mass spectra
204 and NIST spectral library stored in the computer soft-ware (version 1.10 beta, Shimadzu).

205

206 **3. Results and Discussion**

207 **3.1 Cytotoxicity of simulated textile dyeing wastewater during anoxic/aerobic treatment**

208 The growth inhibition of samples IN, AN and AE to yeast cells were shown in Figure 1. It could be
209 seen that sample IN was the most toxic, and its I_c value reached 29.2% even at 100-fold concentration,
210 and was over 50% at 500-fold. The growth inhibition of sample AN was higher than that of sample AE
211 at each concentration, and their I_c values were 30.1% and 16.9% at 500-fold concentration, respectively.
212 These results suggested that cytotoxicity of the simulated textile dyeing wastewater decreased
213 gradually by anoxic/aerobic biological treatment.

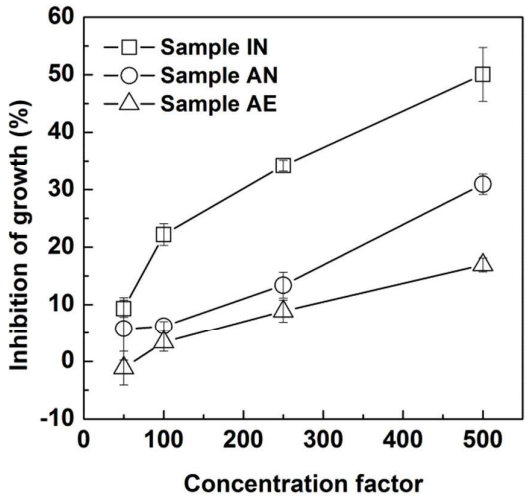

Figure 1. The cytotoxicity of samples IN, AN and AE. Error bars represent the standard deviation based on triplicate analyses.

3.2 Estrogenic/antiestrogenic activities of simulated textile dyeing wastewater during anoxic/aerobic treatement

In this study, antiestrogenic effects were observed in the samples IN, AN and AE, but estrogenic effects were not detected in all samples. To avoid the impact from cytotoxicity, three samples at 50-fold concentration assessed as non-toxic (I_c values < 10%) were chosen to investigate the change of antiestrogenic activity during anoxic/aerobic treatment. As shown in Figure 2, the inhibition of *β*-galactosidase activity decreased moderately after anoxic reaction, which was from 5.7% down to 3.5%, but significantly increased to 18.2% after aerobic reaction ($p < 0.05$). This result suggested that some active substances could antagonize E2 generated during the aerobic biological treatment.

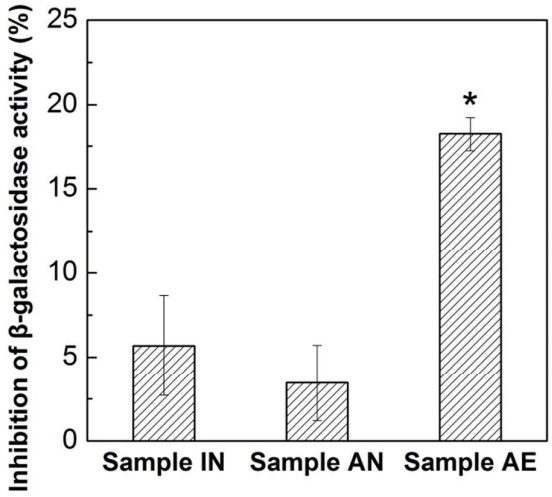

Figure 2. The antiestrogenic activity of samples IN, AN and AE at 50-fold concentration. Asterisk sign (*) indicates that the antiestrogenic activity of sample was significantly different from that of the sample IN ($p < 0.05$). Error bars represent the standard deviation based on triplicate analyses.

Therefore, the simulated textile dyeing wastewater in this study had antiestrogenic activity. It also has been reported that high antiestrogenic activity was detected in industrial effluent from textile and dyeing wastewater treatment plants [7], but not involving the relative study about the characteristics of the antiestrogen-active substances.

3.3 Cytotoxicity of different DOM fractions from the anoxic and aerobic effluent

For analyzing the main antiestrogen-functional component, sample AN and sample AE were fractionated into four fractions, including HOA, HOB, HON and HIS. The inhibition of four fractions from two samples to the growth of yeast cells were shown in Figure 3. HON of each sample had obvious toxic effect on the yeast cell. Even at 250-fold concentration, the I_c values of HON were 13.7% and 11.1% in sample AN and AE, respectively, which was over non-toxic limit. While the other three fractions of each samples were assessed as non-toxic because their Ic values were all less than 10%.

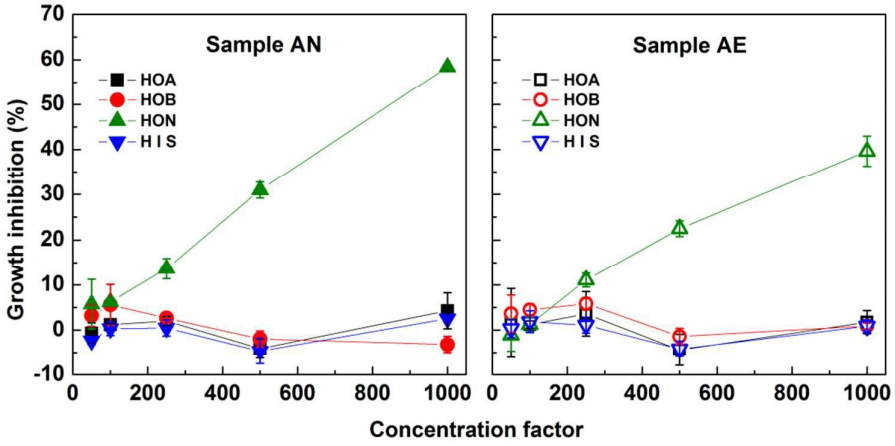

Figure 3. The cytotoxicity of four fractions of the sample AN and sample AE at different concentration factor. HOA, hydrophobic acids; HOB, hydrophobic bases; HON, hydrophobic neutrals; HIS, hydrophilic substances. Error bars represent the standard deviation based on triplicate analyses.

HON was the only fraction with deep color of four DOM fractions in samples AN and AE, which had strong absorbance in visible spectra of 400-700 nm. This phenomenon suggested that there were some colored matters, including undegraded textile dyestuffs and biodegradation products with chromophoric groups, existing in the HON fraction of both samples. It is possible that the cytotoxicity of HON fraction was mainly related to these colored substances.

3.4 Antiestrogenic activity of different DOM fractions from the anoxic and aerobic effluent

From the Figure 4, DOM fractions from samples AN and AE had different antiestrogenic activities. The β-galactosidase activity inhibition of HOB and HIS of both samples did not increased obviously or change regularly along with the increasing concentration factor, which indicated that these two fractions did not elicit obvious antiestrogenic response. But HOA and HON of both samples could cause distinct reduction of β-galactosidase activity. The inhibition of HOA in sample AN to β-galactosidase activity increased from 5.9% at 50-fold concentration to 64.9% at 1000-fold concentration, and that in sample AE it ranged from 7.1% to 84.2%. The HON also had strong antiestrogenic activity in sample AN and AE, and their β-galactosidase activity inhibition reached 21.9%

and 44.7% at 50-fold concentration, respectively. Because the I_c value of HON far exceeded the non-toxic limit at 500- and 1000-fold concentration, the corresponding data were not shown in Figure 4. Overall, the antiestrogenic activity of HOA and HON in sample AE was significantly higher than that in sample AN, which was consistent with antiestrogenic activity between un-fractionated sample AN and sample AE.

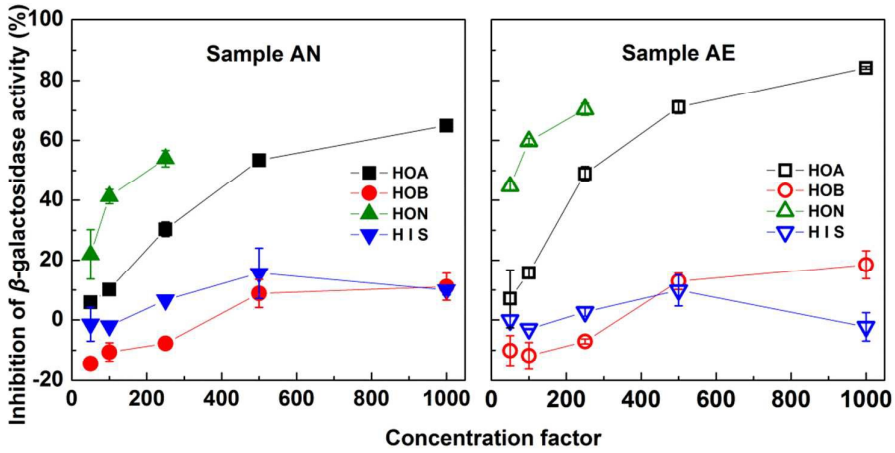

Figure 4. The antiestrogenic activity of four fractions from the sample AN and sample AE at different concentration factor. HOA, hydrophobic acids; HOB, hydrophobic bases; HON, hydrophobic neutrals; HIS, hydrophilic substances. Error bars represent the standard deviation based on triplicate analyses.

Therefore, the main antiestrogen-active fractions were HOA and HON. Until now, a lot of research have studied the mechanisms for antiestrogenic effects, including estrogenic receptor antagonists and/or interaction [32-34], sorption by macromolecules [21, 35, 36], and some other non-specific ways such as changes of membrane permeability for estrogenic chemicals [37]. It has been reported that the antiestrogenic chemicals such as tamoxifen and raloxifene can completely bind to ER and induce a conformational change, which inhibit the transformation of estrogen-dependent genes [33], and result in the antiestrogenic activities. In addition, as in this study, the toxicity of the compound had exceed its putative endocrine effects, and the yeast acted as a toxicity biosensor [34].

HOA faction showed inhibition of *β*-galactosidase induction, but not inhibition of yeast cell growth.

Thus, the antiestrogenic activity of HOA may result from the antiestrogenic chemicals affect the induction mechanism of E2 in yeast such as competitively bind to the ER, and/or the macromolecules, which can absorb the E2. The probability of high inhibition of β-galactosidase activity of HON in both samples is mainly related to the high cytotoxicity. In addition, HON may contain molecules in larger size and micellae such as colloidal organic carbon (COC) which can pass through microfiltration membrane. Thus these organic matters in HON, which can be intercepted by the XAD-8 resin and eluted by Soxhlet extraction, may give rise to stronger E2 sorption behavior [36].

3.5 Excitation-emisson matrix (EEM) fluorescence spectroscopy of different samples and DOM fractions

Samples AN and AE and their fractions exhibited different antiestrogenic activity in this study. Thus, an EEM fluorescence spectroscopy was used to characterize the chemical structures of soluble organic matters in samples. The contour maps of the results were shown in Figures 5, 6 and 7. The peaks of different fractions were related to substances with different chemical structures according to the previous research [38]. The intensity of peak HOB2 recorded was normalized as 1000 arbitrary units (AU). The location of Ex_{max}/Em_{max} and intensity of these peaks and their corresponding substances were listed in Table 2 and 3.

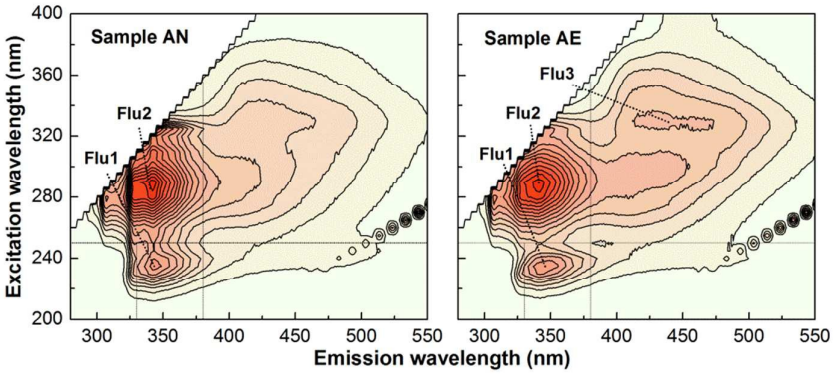

Figure 5. EEM fluorescence spectra for samples AN and AE.

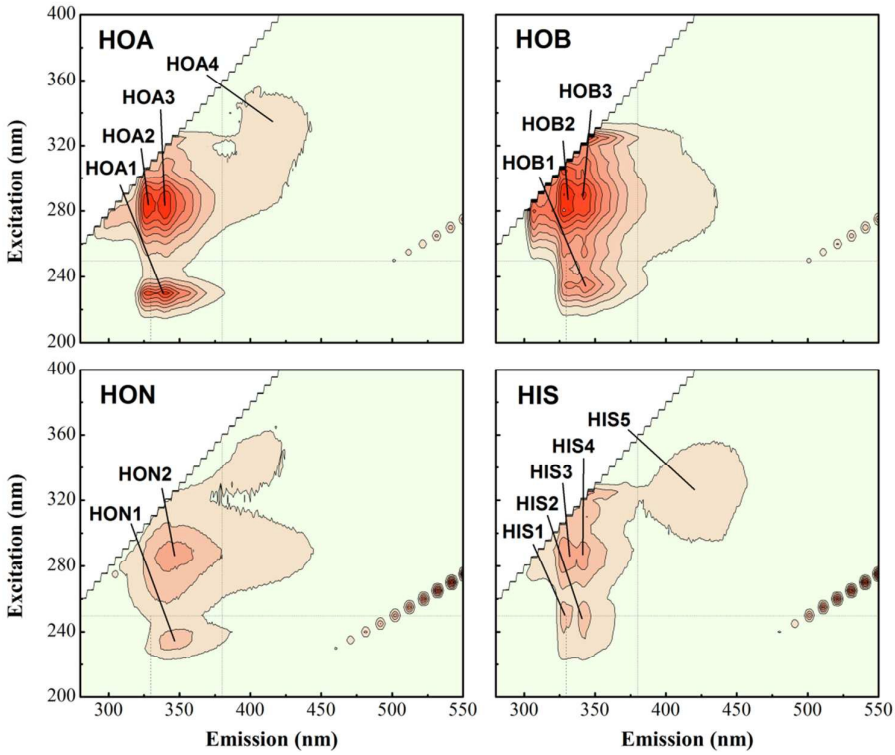

Figure 6. EEM fluorescence spectra for four fractions of sample AN. HOA, hydrophobic acids; HOB, hydrophobic bases; HON, hydrophobic neutral; HIS, hydrophilic substances.

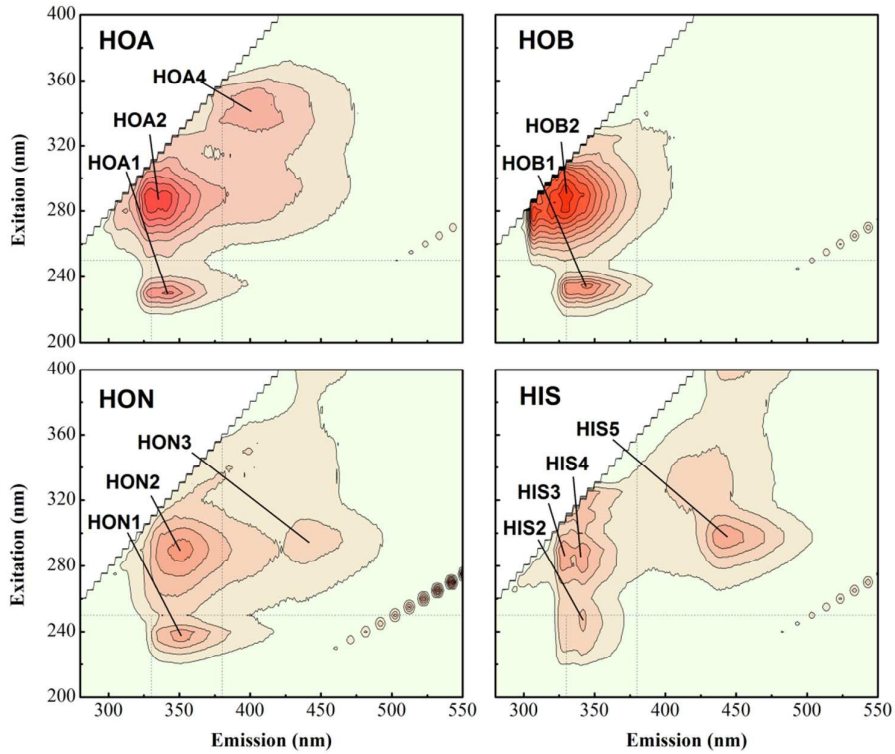

Figure 7. EEM fluorescence spectra for four fractions of sample AE. HOA, hydrophobic acids; HOB, hydrophobic bases; HON, hydrophobic neutral; HIS, hydrophilic substances.

Table 2 EEM peaks for samples AN and AE

Peak	Sample AN Ex_{max}/Em_{max} (nm/nm)	Sample AN Intensity at Ex_{max}/Em_{max} (AU)	Sample AE Ex_{max}/Em_{max} (nm/nm)	Sample AE Intensity at Ex_{max}/Em_{max} (AU)	Homologous substances
Flu1	235/343	906	235/347	747	Tryptophan-like, aromatic protein
Flu2	290/323	1896	290/341	1480	Soluble microbial by-product-like
Flu3			330/434	488	Humic acid-like

Table 3. EEM peaks for the fractions of sample AN and sample AE

Peak	Sample AN Ex_{max}/Em_{max} (nm/nm)	Sample AN Intensity at Ex_{max}/Em_{max} (AU)	Sample AE Ex_{max}/Em_{max} (nm/nm)	Sample AE Intensity at Ex_{max}/Em_{max} (AU)	Homologous substances
HOA1	230/339	506	230/341	250	Tryptophan-like, aromatic protein
HOA2	280/327	530	285/331	355	soluble microbial by-product-like
HOA3	285/341	541			soluble microbial by-product-like
HOA4	325/411	89	335/406	173	humic acid-like
HOB1	235/344	591	235/345	299	Tryptophan-like, aromatic protein
HOB2	280/328	1000	290/328	506	soluble microbial by-product-like
HOB3	290/342	905			soluble microbial by-product-like
HON1	235/348	121	240/352	214	Tryptophan-like, aromatic protein
HON2	285/344	183	290/350	260	soluble microbial by-product-like
HON3			295/438	133	humic acid-like
HIS1	250/328	135			aromatic protein
HIS2	250/342	139	250/342	154	Tryptophan-like, aromatic protein
HIS3	290/327	214	290/327	214	soluble microbial by-product-like
HIS4	290/341	203	290/342	229	soluble microbial by-product-like
HIS5	325/420	94	300/442	226	humic acid-like

These EEM peaks are related to tryptophan-like aromatic protein, soluble microbial by-product-like or humic acid-like organic compounds according to a location of EEM peaks of many typical chemicals in wastewater [38]. From Table 2, it was found that more soluble microbial by-product-like and aromatic protein-like substances existed in sample AN, and more humic/fulvic acid-like substances existed in sample AE. From Table 3, HOB had the highest content of aromatic protein and soluble microbial by-product and HIS had the highest content of humic/fulvic acid both in sample AN and

315 sample AE. However, these two fractions did not elicit obvious antiestrogenic response. The aromatic
316 protein in HOA decreased after aerobic treatment, but the antiestrogenic activity increased. Therefore,
317 it is necessary to find whether some antiestrogen-active substances generated after aerobic reaction.
318 Furthermore, since the content humic/fulvic acids in HOA and HON both increased after aerobic
319 reaction, it is possible that these macromolecules mask the estrogenic activity in the bioassay and
320 exhibit antiestrogenic activity [35, 36].

321 **3.6 Products analysis**

322 In this study, antiestrogenic activity also may arise from the mechanisms such as competitive binding,
323 but not just simple sorption of E2. Some antagonists such as 4-hydroxytamoxifen can competitively
324 bind to ER leading to a conformational change in receptor, and culminate in inhibition of gene
325 expression [33]. Therefore, gas chromatography-mass spectra (GC-MS) analysis was carried out to
326 determine what intermediate and/or degradation products in wastewater samples AN and AE, which
327 might be related to cytotoxicity or estrogenic/antiestrogenic activities. The detected compounds were
328 shown in Supplementary Table 2.

329 In GC-MS determination of sample AN, a lot of low-weight-molecule organic acids were detected
330 by comparison of retention times and mass spectra of standards, including short chain fatty acids
331 (SCFAs) such as propionic acid, butyric acid, valeric acid, caproic acid, and the isomers such as
332 isobutyric acid, 2-/3-methylbutyric acid, and the derivatives such as 2-hydracrylic acid. These organic
333 acids generated by anoxic biodegradation and resulted in low pH of anoxic effluent. Moreover, some
334 aromatic compounds also could be identified, including aromatic acids such as benzoic and
335 phenylacetic acids and phenolics such as phenol, m-cresol and 4-methylcatechol.

It has been reported that some weak acids such as propionic, butyric, caproic and benzoic acids can inhibit the fermentation rate of the *Saccharomyces cerevisiae* [39]. From the study of Wattanadilok et al., the phenylacetic acid had the growth inhibitory effect on test seven yeasts [40]. With regard to the two detected phenolic compounds, m-cresol was the most active cresol isomer in antifungal activity to the *Fusarium verticillioides* [41], and 4-methylcatechol was found to be able to strongly decrease the growth rate of *Debaryomyces hansenii* [42]. These studies indicated that the detected compounds may have inhibitory/cytotoxic effect on the tested yeast *Saccharomyces cerevisiae* Y190.

While less organic acids were detected in the sample AE, which suggested that low-weight-molecule organic acid were degraded under aerobic conditions. Noteworthy, the p-phenetidine and phthalic acid gave peaks at 15.1 and 20.2 min in all the samples. P-phenetidine can be cleaved from direct yellow 12 and assigned to priority substance because it may cause sensitization by skin contact [43]. Recent studies have shown that several fungi strains are able to degrade the direct blue 19 and phthalic acid was identified from the accumulated degradation products [44]. Thus it is possible that the phthalic acid identified in this study may be also generated from the degradation of direct blue 19. Pavan B. et al first demonstrated that phthalic acid can bind to the estrogenic receptor with high affinity and mimics 17β-estradiol actions in WISH cells [45]. Furthermore, recent studies of phthalic acid found that it can cause a general significant increase of vitellogenin (vtg) protein in both sexes and induce significant increase of ERα gene expression [46]. The reason that discrepancy between YES biological assay and GC-MS analysis lie in two aspects: the concentration level of trace target product - phthalic acid, which can be further degraded by aerobic biodegradation, was too low to be detected; the complicated sample may elicit the comprehensive biological effect, but not estrogenic effect.

However, the antiestrogenic chemical has not been identified in this study, which might mainly on account of the complexity and limitation of identification means. Therefore, potential estrogenic/antiestrogenic chemicals should be identified and characterized by more comprehensive detection methods in future study.

4. Conclusions

In this study, the estrogenic/antiestrogenic activities during the anoxic/aerobic treatment were investigated by yeast two-hybrid assay. The results showed that estrogenic activity were not detected during the whole treatment process. However, the antiestrogenic activity decreased via anoxic treatment, but increased significantly after aerobic treatment. Among four fractions, hydrophobic acids (HOA) and hydrophobic neutrals (HON) played important roles in increasing antiestrogenic activity during anoxic/aerobic treatment. In addition, more humic/fulvic acid indicated antiestrogenic activity were found in HOA and HON fractions in aerobic effluent than that in anoxic effluent. In future, more different biotoxicity indicators, such as acute toxicity and genotoxicity of samples are needed to be detected, and the real toxicity of samples are expected to be revealed more comprehensively and completely.

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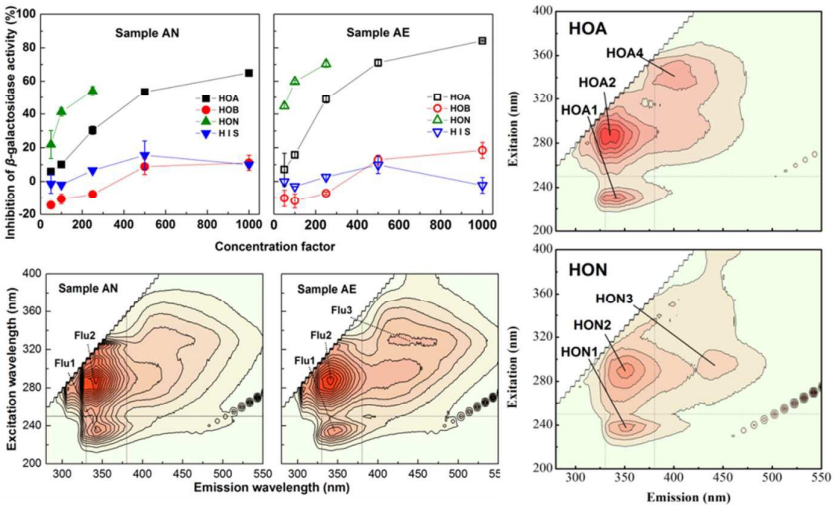
Graphical Abstract



HOA and HON were key fractions involved in increasing antiestrogenic activity and humic/fulvic acid in them could mask estrogenic activity.