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Estrogenic activity of fractionate landfill leacahte

Hua Zhang[a,b], *Cheng-Hsuan Chang*[b], *Fan Lü*[a,b,c], *Duu-Jong Lee*[b,*], *Pin-Jing He*[a], *Li-Ming Shao*[a], *Ay Su*[c]

[a]*State Key Laboratory of Pollution Control and Resources Reuse, Tongji University, Shanghai 200092, China*
[b]*Department of Chemical Engineering, National Taiwan University, Taipei, Taiwan 10617*
[c]*Department of Mechanical Engineering, Fuel-Cell Center, Yuan Ze University, Taoyuan, 320, Taiwan*

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ABSTRACT

Dissolved organic matter (DOM) in leachate samples collected at Laogang Sanitary Landfill in Shanghai, China were fractionated using size exclusion chromatography and were characterized using estrogen receptor-α competitor screening assay as toxicity activity index. The estrogen activity of leachate was determined mainly by the hydrophobic acid DOM with moderate MW (3000–14,000 Da) and low MW (<630 Da) and with high aromaticity and fluorophores of possibly pyrenyl characteristics. Landfilling for up to seven years or oxic storage for one month degraded a few of these estrogens. Aerobic SBR treatments effectively degraded the hydrophobic estrogens, but rarely degraded the hydrophilic estrogens.

1. Introduction

Synthetic toxic contaminants that are released into the environment incur ecological and toxicological problems (Kuster et al., 2005). Leachates from municipal solid waste (MSW) landfills can severely contaminate groundwater and surface waters with natural estrogens and xenobiotic organic compounds, including bisphenol A, nonylphenol, phthalates, PCBs, pesticides, organotin and their derived products (Behnisch et al., 2001; Kjeldsen et al., 2002; Marttinen et al., 2003; Mersiowsky et al., 2001). Quantitative monitoring of all toxic chemicals in real leachate, however, is not practically feasible, because of the extremely high testing time and cost needed (Marttinen et al., 2002; Kuster et al., 2005). Bioassays can be used to characterize the toxic activity of landfill leachate with all of its known and unknown constituents.

The toxicity of a landfill has been examined with reference to its acute or sub-chronic toxicity (due to daphnia, algae, rotifer, crustaceans, protozoa, luminescent bacteria) and genotoxicity (such as due to *Salmonella typhimurium*) (Assmuth and Penttila, 1995; Baun et al., 2000; Castillo and Barceló, 2001; Clément et al., 1997; Isidori et al., 2003; Kjeldsen et al., 2002; Pivato and Gaspari, 2006). Assmuth and Penttila (1995) correlated the lethality of acute toxicity, using *Daphnia Magna* with the concentrations of Cl^-, NH_4^+–N, light metals and other water quality parameters. Baun et al. (2004) reported that the concentration of leachate by a factor of 1.3–9.4 yielded EC_{50} according to both Biotox (*Vibrio fischeri*) and algae (*Pseudokirchneriella subcapitata*) biotests, while leachate that was concentrated tenfold exhibited genotoxicity. Castillo and Barceló (2001) observed that the fraction of leachate that had a log K_{ow} of 1.7–5.16 was the most toxic to *Daphnia Magna*. The concentrations of ammonia and nitrite were correlated with the toxicity of tested leachate (Pivato and Gaspari, 2006; Dave and Nilsson, 2005). Clément et al. (1997) suggested that both ammonia and alkalinity are responsible for the most of the leachate toxicity. Isidori et al. (2003) found that pH, cations, suspended and apolar compounds influenced leachate toxicity.

Since estrogens can detrimentally affect the reproduction of wildlife at concentrations of only a few parts per trillion (ng L^{-1})

* *Corresponding author.*
E-mail address: djlee@ntu.edu.tw (D.-J. Lee).

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(Kuster et al., 2005), their toxicity in leachate is becoming of increasing concern (Behnisch et al., 2001; Coors et al., 2003; Kawagoshi et al., 2003; Kawagoshi et al., 2002; Noaksson et al., 2005) Noaksson et al. (2003) concluded that the endocrine disrupting chemicals detected in some rivers and lakes were from a nearby refuse dump. Endocrine receptor-positive human MCF-7 breast cancer cells (E-screen assay) was adopted to analyze the total estrogenic activity of leachate and the treated effluents (Behnisch et al., 2001). Coors et al. (2003) measured the estrogenic activity of leachate from an MSW landfill using a subline of MCF-7 cells (MVLN cells). Coor et al. established that the estradiol equivalent (EEQ) of raw leachate was 57.7±9.4 ng L^{-1}, mostly contributed to by bisphenol A, while biological treatment (aerobic degradation + ultrafiltration + activated carbon adsorption) or reverse osmosis filtration removed 97% of the estrogenic activity of leachate. Kawagoshi et al. (2003) observed that 22-d aerobic treatment eliminated most of the estrogenic activity of leachate.

The fractionation approach with multiple on-line detectors enriches our understanding of quantitative and qualitative dissolved organic matters (DOM) properties in water (Her et al., 2003). Persson et al. (2006) monitored the transformation of organic matter in leachate using size exclusion chromatography (SEC) and mass spectrometry. They also measured qualitative changes in leachate DOM along a groundwater gradient. To the best of the authors' knowledge, few studies have examined the estrogenic activity of fractionated DOM in landfill leachate.

This work fractionated using SEC approach the DOM in leachate collected at an MSW landfill of different landfill age, and at outlets of subsequent oxic storage or aerobic treatment stage. Then, the DOM in each of the SEC fractions were characterized by UV_{254} absorbance, fluorescence excitation and emission matrices, and the estrogen receptor-α (ER-α) competitor screening assay (ERSA). Detecting distributions of estrogen potentials in different DOM fractions advances our understanding for the complex interactions between estrogens and organic matters, and serves as indices for decontamination process selection and operations.

2. Materials and methods

2.1. The leachate sample

Leachate samples (S3, S4, S6, S7 in Fig. 1) were collected from the Laogang Sanitary Landfill in Shanghai, China. The area-type Laogang landfill for municipal solid waste was constructed in four phases: Phase I (1.6 km^2), II (1.0 km^2), III (0.82 km^2) and IV (3.3 km^2) of the landfill have been operated since 1991, 1996, 2000 and 2005, with daily capacities of 3000, 3000, 1500 and 5000 tons of waste, respectively. Sample S3 was from cells from landfills of age one to two years. Sample S4 was from cells from landfills of age three to seven years. The effluent from landfill cells of age one to two years was sent to the oxic storage pond with a retention time of one month. The overflow from the pond (S6) was sent to an aerobic sequential batch reactor (SBR) for polishing before disposal. Sample S7 was the leachate that was collected at the SBR outlet.

The leachate samples were filtered through a 0.45 μm filter that was made of polypropylene (PP) housing and a hydrophilic polytetrafluoroethylene (PTFE) membrane (MFS-25, Pleasanton, Calif., USA). The filtrates were adjusted to pH 7 and stored at 4 °C before analysis.

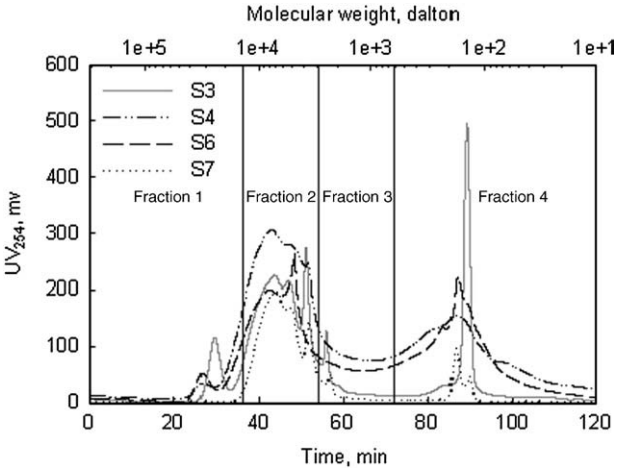

Fig. 1 – The SEC–UV_{254} chromatographs of leachate samples. Four fractions indicated were collected for subsequent estrogenic tests.

In all of tests, potential contamination sources, such as unclean curette, atmospheric aerosols, impure mobile phase or instrumental noise were minimized for data quality assurance. All tests were performed in triplicate with mean and variance presented.

2.2. Chemical analysis

The leachate samples were pretreated with filter paper before analysis. The leachate pH and ORP were measured using a pH/ORP meter (OAKTON Instruments, IL, USA). The carbon and nitrogen content were measured with TN_b/TC multi N/C 3000 Analyzer (Analytik Jena AG, Jena, Germany). Kjeldahl nitrogen (KN), NH_4^+–N, NO_3^-N and total phosphorus (TP) were measured using standard methods. Organic acids (including lactic acid, volatile fatty acids from C1 to C6 as well as iso-C4 and iso-C5) of the 0.20-μm filtered samples were measured by high performance liquid chromatograph (HPLC, Shimadzu Co. Ltd., Japan; post-column pH-buffered electroconductivity method). The HPLC was equipped with a LC-20AD solvent delivery unit for mobile phase, a LC-20AD solvent delivery unit for buffer solution, a CDD-10Avp conductive detector, a CTO-10ASvp column oven, a SCL-10Avp system controller, a Shim-pack SPR-H analyze column and a LC solution workstation.

2.3. Sample preparation

The leachate samples before the ERCA test were concentrated by solid phase extraction (SPE), and were then fractionated by SEC and hydrophilicity (HF) resins.

Samples underwent SPE using OASIS HLB cartridges (6 cm^3, 500 mg OASIS HLB, Waters Corporation, USA). 5 ml of methanol followed by 5 mL of Milli-Q water was used to condition the HLB tubes (Supelco) to analyze the pesticide residue. Then, the quantities of leachate samples that flowed through the tubes were fixed, and then these samples were washed using 5 mL

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Milli-Q. Following drying with nitrogen gas for 30 min, the cartridges were rinsed twice with 5 mL of methanol. The eluted solutions were evaporated under nitrogen gas to dryness. The residues were then dissolved in 0.2 mL of DMSO concentrated by factors of 50 or 100 times. A series of six dilutions (1, 1/5, 1/25, 1/100, 1/500, 1/2500) of the concentrated samples were prepared in DMSO for bioassay analysis.

The HPLC-SEC fractionation system was comprised of BETA 10 Gradient pump (Ecom spol. s r. o., Prague, Czech Republic), a size exclusion HW-50S column (TOYOPEARL resin with a particle size of 20–40 μm, TOSOH Bioscience LLC, Montgomeryville, PA, USA), an on-line SAPPHIRE 600 UV–VIS variable wavelength detector (Ecom spol. s r. o., Prague, Czech Republic) and a CHF 100SA fraction collector (Advantec MFS, Inc., Dublin, CA, USA). The column was adopted at room temperature with a phosphate mobile phase (0.0024 M NaH_2PO_4 + 0.0016 M Na_2HPO_4, pH 6.8) that contained 0.025 M Na_2SO_4, generating an ionic strength of 0.1 M. The flow rate of the mobile phase was 1 mL min^{-1}. 2 mL of the sample was injected, and the effluent was collected over 0–36 min (>14,000 Da), 36–54 min (3000–14,000 Da), 54–72 min (630–3000 Da) and 72–120 min (<630 Da) as fractions 1–4, respectively. Polyethylene glycols (PEGs, 200, 1000, 4000, 8000 and 20,000 Da) were used for the MW calibration of the chromatograms. The absorption wavelength of the UV detector was set to 254 nm. UV data were collected every 0.05 s. The SEC column performed well when each leachate sample was handled five times. 10 mL volumes of S3, S4, S6 and S7 were fractionated.

The procedure that was used to fractionated the organic matters in the leachate samples was modified from that of Leenheer (1981). The Supelite™ resin DAX-8 (Supelco, Sigma-Aldrich, MO, USA) adsorbed hydrophobic substances with different charges (hydrophobic neutral compound: HPON; hydrophobic acid: HPOA; hydrophobic base: HPOB. The resins were all purified by Soxhlet extraction prior to being packed in Φ 2 cm × H 25 cm glass columns (Amersham Biosciences, NJ, USA). The pH of the 0.45-μm filtered leachate samples (containing near 20 mg of DOC) was adjusted to neutral and was pumped through the DAX-8 column. Following adsorption, the Milli-Q water was pumped through the resin to elute any unadsorbed residual liquor. The adsorbed, hydrophobic base (HPOB) on the DAX-8 resin was eluted with 0.1 M HCl. The DAX-8 effluent was then acidified to pH 2 with 6 M HCl and was sent to the DAX-8 column to adsorb the HPOA and HPON fractions, which were eluted sequentially using 0.1 M NaOH and pure or 40% (v/v) methanol. Vacuum-rotary evaporation at 40 °C removed methanol from the HPOIN extract.

The dissolved organic carbon (DOC) contents of 2 mL of each fraction were measured (Aurora Model 1030 TOC Analyzer, OI Analytical, College Station, TX, USA).

2.4. EEM analysis

Fluorescence EEM spectra of 3 ml samples were measured by a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA). EEM spectra were collected with subsequent scanning emission spectra from 250 to 600 nm at 2 nm increments by varying the excitation wavelength from 200 to 500 nm at 10 nm increments. The spectra were recorded at a scan rate of 1200 nm/min, using excitation and emission slit bandwidths of 5 nm. The voltage of photomultiplier tube (PMT) was set at 800 V for low level light detection. Second-order Rayleigh and Raman scattering were filtered out by filters. The cuvettes, Milli-Q water, effluent of mobile phase from SEC were scanned for each batch of fluorescence analysis, to avoid potential pollution during operation. The cuvettes were rinsed and ultrasonicated with 5% (w/w) nitric acid solution before every scan. The EEM contour was produced by Cary Eclipse Software 1.1 (Varian Inc., Palo Alto, CA, USA).

2.5. Estrogen receptor-α competitor assay

An Estrogen Receptor-α (ER-α) Competitor Screening Kit (Wako Pure Chemical Industries, Ltd., Japan) was used to analyze the estrogenic activities of the leachate samples. The assay was conducted according to the protocol. 5 μL of the sample and 95 μL of the reaction solution were reacted for 2 h at room temperature in the microplate well that was coated with ER-α. Then, the mixture was discarded and the wells were rinsed twice by 200 μL of wash solution. 100 μL of the assay solution was added to dissolve the coated ER-α, and analyzed in a Multi-Detection Microplate Reader (Synergy HT, BioTek, USA) at Ex/Em = 485 nm/535 nm. Two wells without any coating were used as the blank. The inhibition ratio was calculated using

$$\text{Inhibition ratio (\%)} = 100 - \frac{(\text{fluorescent intensity of the sample}) - (\text{fluorescent intensity of the blank})}{(\text{fluorescent intensity of pure DMSO}) - (\text{fluorescent intensity of the blank})} \times 100\%$$

3. Results

3.1. Leachate characteristics

Table 1 lists the physiochemical characteristics of all of the samples. The leachates S3–S7 had similar TOC and IC levels,

Table 1 – Physiochemical characteristics of leachate samples

Samples	Unit	S3	S4	S6	S7
pH		8.02	7.8	7.97	8.62
ORP	mV	−75	−217	−45	10
TOC	mg L^{-1}	2350		2430	1290
IC	mg L^{-1}	2750	1790	2620	3430
TC	mg L^{-1}	5100	5270	5050	4710
TN	mg L^{-1}	4470	3680	4320	1650
NH_4^+–N	mg L^{-1}	2150	1740	2060	66
NO_3^-N	mg L^{-1}	3210	136	2950	157
KN	mg L^{-1}	2440	1980	2360	57
TP	mg L^{-1}	13.3	13.6	12.4	7.74
Lactate	mg L^{-1}	0	0	0	0
Formate	mg L^{-1}	0	0	0	0
Acetate	mg L^{-1}	288	1320	739	0
Propionate	mg L^{-1}	225	408	0	0
iso-Butyrate	mg L^{-1}	0	0	0	0
Butyrate	mg L^{-1}	0	211	0	0
iso-Valerate	mg L^{-1}	0	0	0	0
Valerate	mg L^{-1}	0	352	0	0
Hexylate	mg L^{-1}	0	0	0	0

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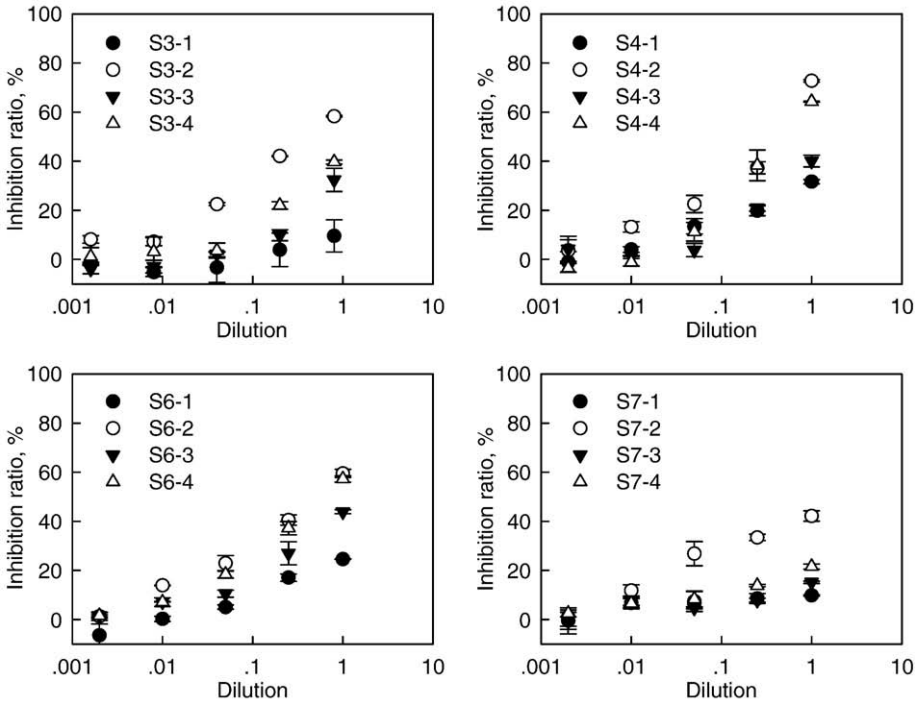

Fig. 2 – Estrogenic activities of SEC-fractionated leachate samples.

and those from the MSW piles exhibited large amounts of volatile fatty acids and ammonia. Oxic storage did not cause the water quality parameters of sample S6 to differ substantially from those of S3. However, aerobic SBR treatment substantially reduced the ammonia level and removed most VFAs (S7).

The S3 (1–2 yr landfill age) and S4 (3–7 yr landfill age) had a peak MW of 200–300 Da and other peak MW values of 2×10^3–2×10^4 Da (Fig. 1). The S3 had considerable levels of UV_{254}-absorbing substances of MW 1.5–5×10^4 Da. The overall UV_{254} absorbance was lower in S3 than in S4. Restated, the average aromaticity of residual compounds increased with landfill age.

The UV absorbance in S3 over MW 1.5×10^4–5×10^4 fell following oxic storage (S6). The SBR treatment eliminated the UV absorbance of MW 1.5×10^4–3×10^4 Da and that of 200–300 Da, but, in contrasting, increased the UV absorbance over an MW range of 4000–20,000 Da (S7).

3.2. *Estrogenic activities of leachates*

The estrogenic activities follow the orders, S4>S3, S6>S7 (Fig. 2), revealing that neither prolonged landfilling nor oxic storage reduced the estrogenic activity of leachate. Conversely, the aerobic SBR treatment substantially reduced the estrogenic activity of the sample. Fig. 2 also shows that the estrogenic activities follow the order fraction 2>fraction 4>fraction 3>fraction 1. Aerobic treatment reduced the activities of DOM in all four fractions.

Fig. 3 displays the estrogenic activities of samples S3 and S7 of hydrophobicity-based fractions. The trend was HPOA>HPON>HPOB and HPI. Restated, the hydrophobic acid fraction

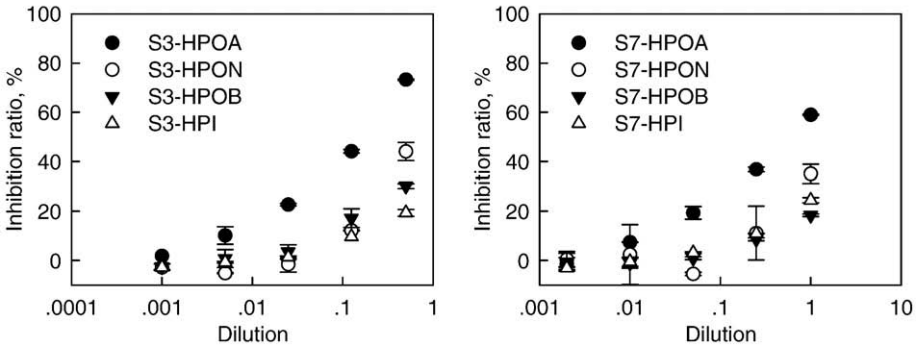

Fig. 3 – Estrogenic activities of resin-fractionated leachate samples.

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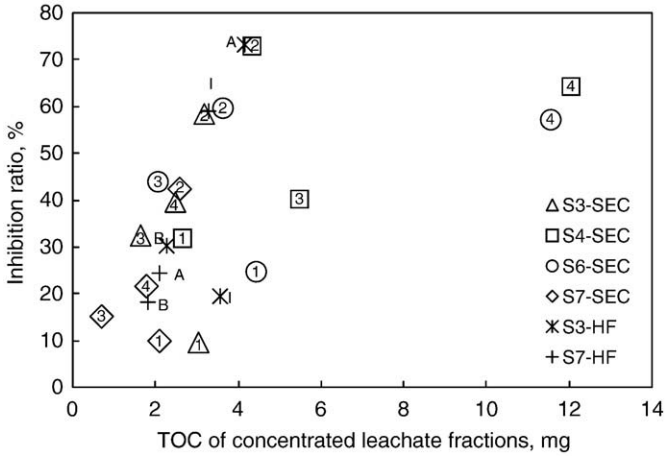

Fig. 4 – Relationship between TOC and estrogenic activity of leachate samples. The numbers in the legends denote the SEC fractions (1–4) of leachates. Letters A, B, and I denote HPOA, HPOB, and HPI fractions, respectively. SEC indicates samples fractionated with SEC, while HF the samples fractionated by resins.

had a higher estrogenic effect than other fractions. SBR treatment reduced the TOC levels of HPOA and HPOB decreased by 60%, and the HPI level by 70%. The corresponding estrogen activities of HPOA, HPON and HPOB were reduced, but those of HPI were not markedly changed.

4. Discussion

4.1. *Correlation with leachate characteristics*

Fig. 4 plots the estrogenic activity data and the corresponding TOC values for the fractionated leachate samples. The estrogenic activity increased with TOC values, but not linearly.

The UV_{254} absorbance data for each fraction displayed in Fig. 1 were integrated with respect to elution time, yielding $\int_{t_1}^{t_2} UV_{254}dt$. Fig. 5 plots the estrogenic activity against this integrand. A close correlation between the estrogenic activity and the integrated UV_{254} data is noted. Log–log regression of the presented data reveals the following statistical results; $R^2=0.983$; Durbin–Watson statistic 1.977 passed; normality test passed ($P=0.851$); K–S statistic=0.147, significance level=0.851; constant variance test passed ($P=0.943$). Accordingly, regardless of the age of the landfill or whether the sample is oxically stored or aerobically treated, estrogenic activity correlated with the quantities of highly aromatic compounds.

Fig. 6 displays the EEM spectra of the tested leachates. A few characteristic EEM peaks were noted, including those at Ex/Em=220/310–340, 220–240/420–460, 310–330/410–450, and those at approximately 270/300 and 280/330 nm/nm. As Fig. 6 reveals, the EEM peaks at Ex/Em=230–440 and 320/410 nm were obtained from all fractionated leachates of high estrogenic activities (such as those in fraction 2 and in the HOPA fractions). Fig. 7a and b plot the estrogenic activity versus the intensities of the EEM peaks at Ex/Em=270/300 and 310/410 nm/nm, respectively.

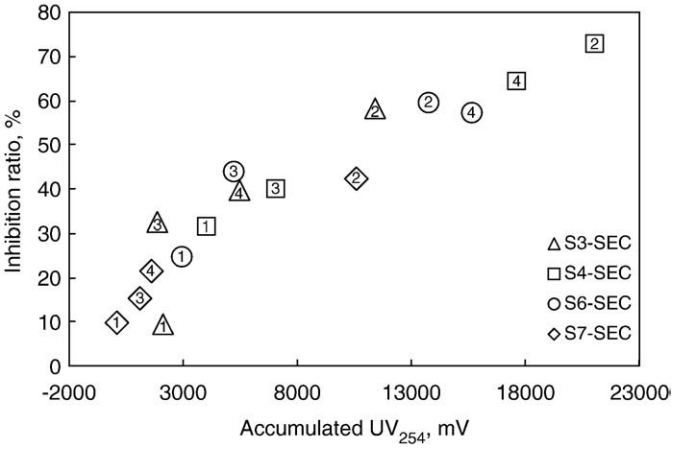

Fig. 5 – Relationship between UV_{254}-t integrand and estrogenic activity of leachate samples. The numbers in the legends denote the SEC fractions (1–4) of leachates.

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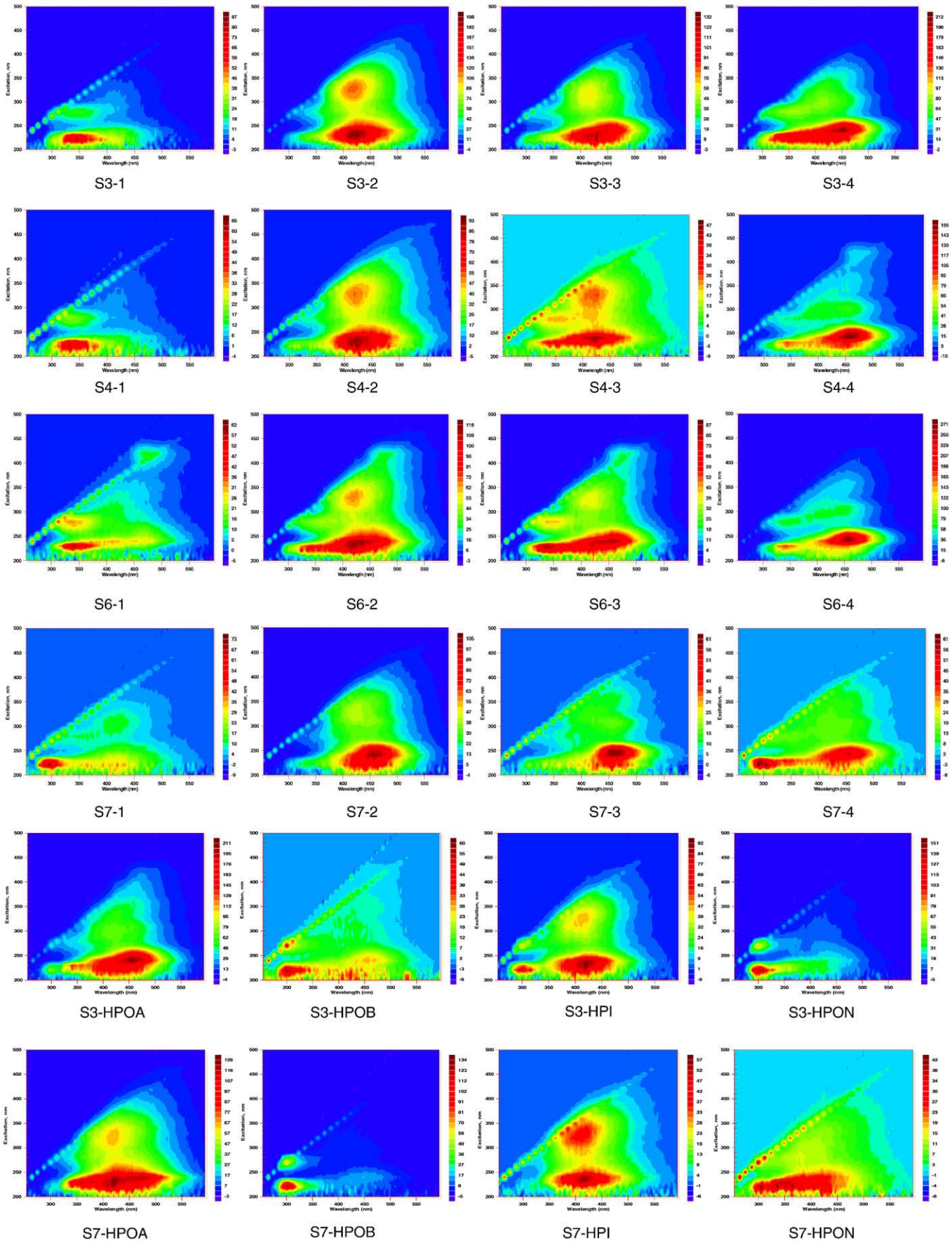

Fig. 6 – EEM spectra of the fractionated leachates.

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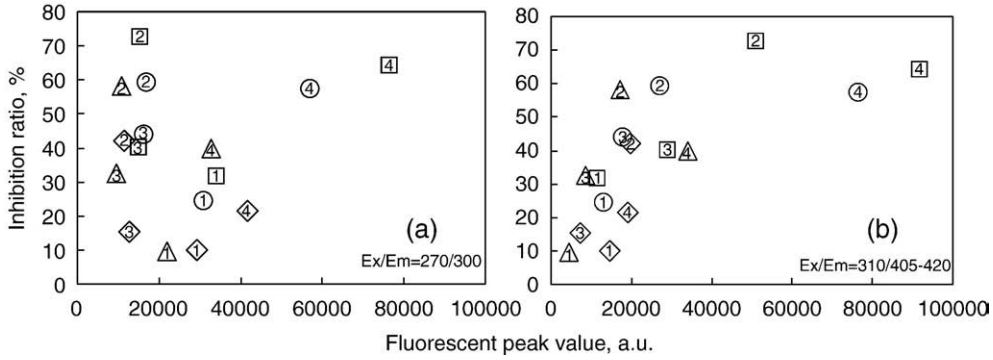

Fig. 7 – Relationship between EEM peak intensities and the estrogenic activities of the leachate. The numbers in the legends denote SEC fractions of leachates.

The data in Fig. 7a show no significant correlation (R^2=0.028); while those in Fig. 7b reveal a good correlation, with the following statistics; R^2=0.929; Durbin–Watson statistic=1.71 (passed); normality test P=0.995 (passed); K–S statistic=0.0999, significance level=0.995; constant variance test P=0.721 (passed).

Table 2 lists the regression coefficients obtained by the log–log regression analysis of estrogenic inhibition ratio against EEM peak intensity of all leachate samples (N=24). The EEM peak intensities at Ex/Em=310/405–420 and 445–460, and 330/410–430 nm/nm correlate significantly with the estrogenic activity of the leachates at the 0.01 level, whereas those at Ex/Em=220–230/375–390, 280/345–365 and 220–240/415–435 and 450–465 nm/nm are correlated with the estrogenic activity at the 0.05 significance level.

4.2. *Characteristics of estrogens in leachate*

The variables that determine the projection (VIP) are evaluated to estimate the relative importance of each EEM peak intensity to the estrogenic activity of leachate. The VIP plot in Fig. 8 demonstrates that the compounds with fluorophores of Ex/Em=(310/410–460 nm/nm), followed by those of 220–240/410–450 nm/nm, are the most important to the estrogenic activity of leachate. According to the classification scheme of Her et al. (2003), these fluorescence characteristic peaks correspond to the humic-like and fulvic-like substances, respectively. The leachates of Baker and Curry (2004) yielded characteristic EEM peaks at Ex/Em=220–230/340–370 and 320–360/400–470 nm/nm. These characteristic peaks correlate with the present estrogen peaks with some wavelength shifts. Compounds with pyrenyl groups could be characterized with excitation peaks close to at 240, 260–280 and 325–350 nm. Coors et al. (2003) concluded that bisphenol A principally contributed to the estrogenic activity in the raw and treated leachate from a municipal waste landfill in Germany. However, since the DOM herein probed have an AMW of 200–50,000 Da, a comparison with Coors et al. results is not feasible.

Table 2 – Correlation coefficients between EEM peak intensities and inhibition ratios

Ex/Em	220/290–305	220–230/335–355	220–230/375–390	220–240/415–435	240/450–465	270/300
R	.0979	0.384	0.510[a]	0.577[a]	0.510[a]	0.172
Ex/Em	280/340	280/345–365	310/405–420	310/45–460	330/410–430	
R	0.419	0.504[a]	0.698[b]	0.652[b]	0.790[b]	

[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

5. Conclusions

This work fractionated using SEC approach the DOM in leachate collected at an MSW landfill of Shanghai, China, and evaluated the estrogenic activities of the fractionated leacahte. The estrogenic activity test revealed that neither prolonged landfilling nor oxic storage reduced the estrogenic activity of leachate. On the other hand, aerobic SBR treatment substantially reduced the estrogenic activity of the sample. Fractionation tests revealed that the hydrophobic acid DOM with MW of 3000–14,000 Da and <600 Da determined most of the estrogen activity of leachate. The estrogenic activity increased with TOC values and total UV_{254} absorbance of fractionated leachate. The EEM peaks at approximately Ex/

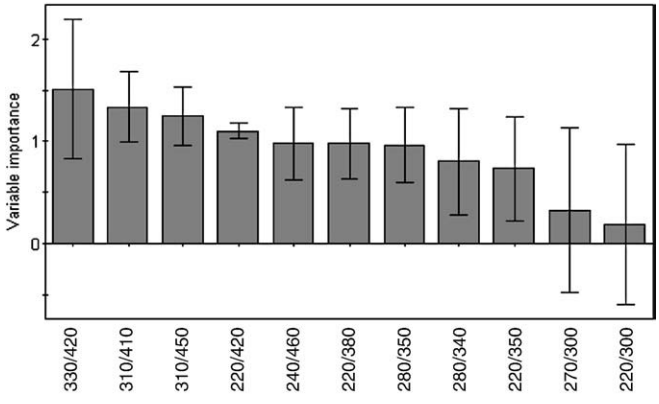

Fig. 8 – Variable importance plot (VIP) computed for EEM peak data.

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Em = 230/410–460 and 310/410–460 nm were obtained from all fractionated leachates of high estrogenic activities. The present estrogens were with high aromaticity and fluorophores of possibly pyrenyl characteristics.

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