Changes in the components and biotoxicity of dissolved organic matter in a municipal wastewater reclamation reverse osmosis system

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ABSTRACT

The characteristics of dissolved organic matter (DOM) and the biotoxicity of these components were investigated in a municipal wastewater reclamation reverse osmosis (mWRRO) system with a microfiltration (MF) pretreatment unit. The MF pretreatment step had little effect on the levels of dissolved organic carbon (DOC) in the secondary effluent, but the addition of chlorine before MF promoted the formation of organics with anti-estrogenic activity. The distribution of excitation emission matrix (EEM) fluorescence constituents exhibited obvious discrepancies between the secondary effluent and the reverse osmosis (RO) concentrate. Using size exclusion chromatography, DOM with low molecular weights of approximately 1.2 and 0.98 kDa was newly formed during the mWRRO. The normalized genotoxicity and anti-estrogenic activity of the RO concentrate were 32.1 ± 10.2 μg4-NQO/mgDOC and 0.36 ± 0.08 mgTAM/mgDOC, respectively, and these values were clearly higher than those of the secondary effluent and MF permeate. The fluorescence volume of Regions I and II in the EEM spectrum could be suggested as a surrogate for assessing the genotoxicity and anti-estrogenic activity of the RO concentrate.

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Introduction

Municipal wastewater reclamation and reuse has been considered a viable and effective approach to solving water shortages in many countries. Reclaimed water has been used in industrial manufacturing, agricultural irrigation, urban greening, rivers and lakes, and groundwater recharge.[1,2] However, municipal wastewater includes not only conventional chemical pollutants but also a variety of toxic-risk chemical pollutants, such as disinfection by-products (DBPs), endocrine disruptors, pharmaceuticals, and personal care products.[3,4] Recently, municipal wastewater reclamation reverse osmosis (mWRRO) has been applied extensively as a treatment method to produce high-quality reclaimed water for specific purposes, such as indirect drinking water, industrial manufacturing, and groundwater recharge.[5–7]

An mWRRO system often uses biologically treated wastewater from municipal wastewater plants as its influent and employs microfiltration (MF) or ultrafiltration (UF) as part of pretreatment prior to reverse osmosis (RO). Furthermore, chemical agents, such as acidifier, disinfectant, antiscalant, reductant, non-oxidizing biocide, or neutralizer, are added during pretreatment prior to RO to ensure the stable and optimal operation of the mWRRO process. mWRRO has high removal efficiencies for inorganic ions, hardness, organic matter, nitrates, ammonia, pathogenic microorganisms, and some trace organic substances, such as DBPs, endocrine disruptors, drugs, and personal care products. Thus, RO permeates can even satisfy the water quality requirements for many potable and non-potable reuse applications.[8,9]

However, although possessing high water quality of RO permeates, mWRRO systems produce a waste stream of RO concentrates containing high concentrations of dissolved salts and recalcitrant organics. The chemical pollutants in the RO concentrate have been affected by not only the water quality of the secondary effluent (the influent of mWRRO) but also the injection of chemical agents during mWRRO. The injection of a non-oxidizing biocide (Kathon biocide) in an mWRRO system has been reported to lead to high in-vitro genotoxicity and can contribute to the higher genotoxicity of RO concentrates.[10] Anti-estrogenic activity was also detected in RO concentrates in our previous research.[11]

Previous studies have investigated the removal of dissolved organic matter (DOM) (dissolved organic carbon (DOC), chemical oxygen demand (COD), and UV254) via...
mWRRO, but the fate of the specific DOM components during mWRRO has not yet been reported. Several studies have evaluated the potential for the removal of acute toxicity, genotoxicity, or retinoic acid receptor activity in the reclaimed municipal treated effluent using different wastewater reclamation technologies, including the RO process.[12,13] However, changes in the anti-estrogenic activity in these mWRRO systems have not been investigated to date. Due to the increasing need for high-quality reclaimed water produced by mWRRO, the fate of organics and the biotoxicity of the process should be investigated.

The aim of this study was to evaluate the characteristics of DOM and the biotoxicity during mWRRO. The changes in the DOM components in the water samples during mWRRO were characterized by excitation emission matrix (EEM) fluorescence spectroscopy and size exclusion chromatography. The biotoxicity was evaluated by genotoxicity and anti-estrogenic activity tests. Additionally, key molecular-size fractions contributing to the biotoxicity of the RO concentrate were investigated.

Materials and methods

The mWRRO and water samples

Water samples were collected from an mWRRO system at a wastewater reclamation plant located in Beijing. The treatment capacity of this plant was 21,000 m$^3$/d (Figure 1). The RO recovery ratio (RO permeate volume per RO influent volume) of the RO system was 60–70%; therefore, the RO concentration ratio (RO influent volume per RO concentrate volume) was in the range of 2.5–3.3. The MF filter was equipped with polyvinylidene fluoride hollow fiber membranes, which had a membrane flux, available membrane area, and nominal filter fineness of 40–100 L/m$^2$ h, 50 m$^2$, and 0.1 µm, respectively. The RO system was equipped with cross-linked aromatic polyamide composite membranes that had a membrane flux, available membrane area, and nominal filter fineness of 17–24 L/m$^2$ h, 34 m$^2$, and 0.1 nm, respectively. More details of the mWRRO system and corresponding chemical agents’ addition have been described in the previous study.[10,11]

The influent of the mWRRO system was the secondary effluent from a sequencing batch reactor that treats municipal wastewater. Water samples of the secondary effluent, MF permeate, RO permeate, and RO concentrate were taken in sequence from A to D (water sampling sites) along the mWRRO system (Figure 1). The MF permeate (Sample B) was stored temporarily in the middle water tank and sampled after storage. The water was sampled monthly from March 2013 to July 2013. All of the water samples were transported to the laboratory, filtered immediately with glass fiber filters (0.45 µm) to eliminate suspended solids, and stored at 4°C to minimize changes in the constituents of the water.

Water quality analysis

The concentrations of COD were analyzed according to standard methods within 24 h of sampling.[14] UV-visible spectrophotometric analyses were carried out with a UV-2401PC UV–VIS recording spectrophotometer (Shimadzu, Japan). The concentration of ammonia nitrogen (NH$_4^+$-N) was analyzed by an ammonia medium range analyzer (HI84185, Hanna, Italy). The concentration of DOC and total nitrogen (TN) were detected with a total organic carbon (TOC) analyzer (TOC-5000A, Shimadzu, Japan). Total dissolved solids (TDS) were detected by a TDS meter (Model SX-650).

Fluorescence spectra

The EEM fluorescence spectra of the water samples were recorded on a fluorescence spectrophotometer (F-2500, Hitachi, Japan). Three-dimensional spectra were obtained by repeatedly measuring the emission spectra in the range of 240–600 nm and at excitation...
wavelengths from 220 to 450 nm. The three-dimensional plots and contour maps were produced using the OriginPro 7.5 program. All of the contour maps were plotted using the same range of fluorescence intensities and numbers of contours. The EEM spectra were divided into different regions at certain excitation and emission wavelengths, associated with tyrosine-like aromatic protein (Region I), tryptophan-like aromatic protein (Region II), fulvic-like (Region III), soluble microbial by-product-like (Region IV), or humic-like organic compounds (Region V).[15]

**Size exclusion chromatography**

Size exclusion chromatography (SEC) was used to characterize the molecular weight (MW) distribution of the water samples. A high performance liquid chromatography (HPLC) (LC20A, Shimadzu, Japan) system with a SPD-M20A UV detector and two connected columns (a TSK-GEL G3000PWXL column followed by a TSK-GEL G2500PWXL column) were employed. The SPD-M20A UV detector was used to determine the UV absorbance at 254 nm. The column temperature was maintained at 40°C, and the injection volume was 100 μL. The mobile phase was composed of Milli-Q ultrapure water buffered with phosphate (0.3744 g/L NaH₂PO₄·2H₂O, 0.5728 g/L Na₂HPO₄·12H₂O) and sodium sulfate (3.55 g/L Na₂SO₄). The relationship between MW and retention time was obtained using MW standard materials that were composed of polyethylene glycol (330, 700, 1,050, 5,250, 10,225, and 30,000 Da) and acetone.

**Fractionation by UF**

To identify the key biotoxic organic constituents of the RO concentrate, water sample was fractionated into different molecular-size fractions by a pressurized stirred-cell apparatus (Millipore Amicon Model-8200) with UF cellulose membrane. The UF membranes, with membrane sizes of 10, 5, 3, and 1 kDa according to the results of the SEC size fraction analyses, were prewashed with Milli-Q water. During UF, five molecular-size fractions of <1, 1–3, 3–5, 5–10, and >10 kDa were obtained. Thereafter, all fractions were diluted to the original volume (500 mL) for further analyses and bioassays.

**Sample concentration for biotoxicity assay**

The water samples were concentrated using Oasis Hydrophilic-Lipophilic Balance (HLB) resin cartridges (Waters Corporation, America) before the genotoxicity and anti-estrogenic activity assay. Each 500 mL water sample was acidified to a pH of 2.0 with 2 M H₂SO₄ and then passed through an Oasis HLB resin cartridge. These cartridges were previously washed with 10 mL of methanol and 10 mL of ultrapure water. Organics that were retained on the cartridge were eluted with acetone and completely dried under a nitrogen flow. The dry residues were then dissolved in 0.5 mL of dimethylsulfoxide (DMSO) to concentrate the components 1000-fold (wastewater volume per the extract volume) for the genotoxicity and anti-estrogenic activity assay.

**Biotoxicity assay**

The SOS/umu assay for genotoxicity was conducted with *Salmonella typhimurium* TA1535/pSK1002 without S9 activation in accordance with ISO 13829.[16] The genotoxicity evaluation method was conducted according to previously described methods.[17] The DMSO solutions with different concentrations of 4-nitroquinoline-N-oxide (4-NQO) were used as positive controls to obtain the dose–response curve of 4-NQO. The genotoxicity was standardized to an equivalent 4-NQO concentration.

The ability of the concentrated sample to inhibit the β-galactosidase activity of estradiol (E2) was measured to determine the anti-estrogenic activity according to the yeast two-hybrid assay. An anti-estrogenic standard chemical tamoxifen (TAM) was used. The inhibitory effect of the concentrated sample and TAM on the β-galactosidase activity that was induced by E2 was measured, and the dose–response curves of the sample and the TAM standard were generated.[18] Thus, the anti-estrogenic activity was recorded as TAM equivalents.

**Results and discussion**

**Performance of the mWRRO**

Water quality characteristics of the secondary effluent, MF permeate, RO permeate, and RO concentrate were given in Table S1 (in Supplementary Information). The TDS in the secondary effluent was 1.19 g/L, without any obvious changes after the MF device; however, over 97% of the TDS was removed by the RO membrane and retained in the RO concentrate (average value of 3.97 g/L). The average concentrations of the COD, DOC, TN and NH₄⁺–N in the secondary effluent were 72.8, 11.4, 17.7 and 0.65 mg/L, respectively. The efficiencies of the mWRRO for removing COD, DOC, UV₂₅₄, TN and NH₄⁺–N in the secondary effluent were 95%, 96%, 92.8%, 93% and 100%, respectively. However, the MF hardly contributed to the removal of contaminants in the secondary effluent, which were mostly separated...
by the RO membrane. Thus, the contaminants in the secondary effluent were transformed into the RO concentrates. Compared with those RO brines from seawater reverse osmosis or brackish water reverse osmosis,\[7\] the RO concentrates from mWRRO exhibited a higher organic pollutant load, but lower salinity.

**Changes in the fluorescence characteristics during mWRRO**

The changes in the fluorescence components of DOM during the mWRRO were characterized using EEM fluorescence spectroscopy (Figure 2). Additionally, the EEM volumes of each region for each wastewater sample were calculated (Table S2 in Supplementary Information) according to the literature.\[15\] The secondary effluent and MF permeate presented a similar EEM spectral shape and paralleled distribution of the EEM volumes of each region.

The RO permeate displayed a weak fluorescence intensity because of the RO membrane, whereas the RO concentrate contained considerably higher concentrations of tryptophan-like aromatic proteins, soluble microbial by-product-like compounds, and fulvic/humic acid-like organic compounds than the other water samples. Although most organics in the secondary effluent were transformed into the RO concentrate, the EEM volume concentration ratio by RO membrane (the EEM volume in the RO concentrate per that volume in the RO influent) of each region was considerably different and those of Regions IV and V complied with the RO concentration ratio. The EEM volume concentration ratio of Region I (represents tyrosine-like aromatic proteins) was at a minimum and slightly higher than those in the secondary effluent and MF permeate. This phenomenon might have occurred because the aromatic protein was hydrophobic, had a smaller MW,\[19\] and preferentially adhered to the RO membrane surface rather than being retained in the RO concentrate. The result also could indicate that RO membrane does not concentrate everything in the secondary effluents and changes the organic components of the secondary effluents.

**Figure 2.** Fluorescence characteristics of the wastewater samples during mWRRO. Region I, tyrosine-like aromatic protein; Region II, tryptophan-like aromatic protein; Region III, fulvic acid-like organic compounds; Region IV, soluble microbial by-product-like organic compounds; and Region V, humic acid-like organic compounds.
Changes in the MW distribution during mWRRO

Based on SEC, the MW distributions of the wastewater samples during mWRRO were obtained within retention times from 28 to 40 min (Figure 3). Specific absorbance regions were identified at the following points according to the UV 254 peaks at the retention time: P1 (approximately 5.1 kDa), P2 (approximately 4 kDa), P3 (approximately 2.9 kDa), P4 (approximately 1.2 kDa), P5 (approximately 0.98 kDa), and P6 (approximately 0.5 kDa). The UV254 absorbance intensity of P3, corresponding to an MW of approximately 2.9 kDa, was the largest peak for each wastewater sample; however, the RO permeate had only one peak at an MW of approximately 2.9 kDa, and the UV254 absorbance intensity for this retention time was sufficiently low to be neglected.

No significant changes in the UV254 absorbance intensities of the specific absorbance regions were detected between the secondary effluent and MF permeate. The specific absorbance regions P1, P2, and P3 were present in the secondary effluent, MF permeate, and RO concentrate; however, the respective UV254 intensities of P1, P2, and P3 of the RO concentrate were three times higher than those of the other water samples. Moreover, P4 and P5 emerged in the RO concentrate but were not present in the MF permeate, which indicated that DOM with low MWs of approximately 1.2 and 0.98 kDa formed in the mWRRO system. This phenomenon also confirmed that the DOM in the RO concentrate was influenced by not only the secondary effluent but also the injected chemical agents and some oxidation – reduction reactions during mWRRO. [10,20]

Changes in biotoxicity during mWRRO

Figure 4(a) presents the changes in concentration of genotoxicity and anti-estrogenic activity of the mWRRO process. The average genotoxicity of the secondary effluent was 261.6 μg-4-NQO/L, which was close to that of the MF permeate (252.4 μg-4-NQO/L). The secondary effluent was chlorinated (1.36–1.70 mg Cl2/L) before the MF to inhibit any bacterial proliferation, but Wu et al. had proved that chlorination under a low NH4–N content could decrease the genotoxicity of secondary effluent. [17] Although the MF permeate was sampled after the middle water tank, to which 3 mg/L antiscalant (primarily

![Figure 3. Changes in the MW distribution during mWRRO. A, secondary effluent; B, MF permeate; C, RO permeate; and D, RO concentrate.](image)

![Figure 4. Changes in the genotoxicity and anti-estrogenic activity during mWRRO. Plus signs indicate that the genotoxicity and anti-estrogenic activity of the water sample are below the detection limit by 1000-fold.](image)
consisting of 2-phosphomobutane-1,2,4-tricarboxylic acid (PBTCA) and an appropriate amount of reductant (NaHSO3) were added, previously report showed that the antiscalant (PBTCA) and complex organic compounds formed through the reactions between the antiscalant and other substances were not genotoxic.[10]

In contrast to the distribution of genotoxicity, the anti-estrogenic activity of the MF permeate was slightly higher than that of the secondary effluent, possibly due to newly formed DBPs. The secondary effluent contained aromatic amino acids and humic/fulvic acids, which are precursors of anti-estrogenic DBPs.[18,21] Moreover, DBPs resulting in anti-estrogenic activity could be formed during the chlorination step in the pretreatment of the mWRRO system.[20,22]

Fortunately, both the genotoxicity and anti-estrogenic activity of the RO permeate were below the detection limit. However, the average genotoxicity and anti-estrogenic activity of the RO concentrate were 1,180.6 μg 4-NQO/L and 13.2 mg TAM/L, respectively. The concentrations of both genotoxicity and anti-estrogenic activity in the RO concentrate were over fourfold higher than those in the MF permeate. Such a value was higher than the RO concentration ratio (2.5–3.3) calculated according to the RO system recovery (60–70%).

Figure 4(b) presents the normalized genotoxicity and anti-estrogenic activity by the corresponding DOC value of the water samples. The genotoxicity and anti-estrogenic activity per mg DOC of the RO concentrate were 32.1 ± 10.2 μg 4-NQO/mg DOC and 0.36 ± 0.08 mg TAM/mg DOC, respectively, which were the highest among the water samples. Additionally, the anti-estrogenic activity per mg DOC in the MF permeate was higher than that of the secondary effluent, which indicated that the addition of chlorine before MF promoted the formation of organics with anti-estrogenic activity. Furthermore, the RO influent was disinfected weekly for 1 h using a non-oxidizing biocide (Kathon biocide, 150 mg/L), which was composed by 5-chloro-2-methyl-4-isothiazoline-3-one (CMIT) and 2-methyl-4-isothiazoline-3-one (MIT) achieving their biocidal activity by reacting with thiol-containing enzymes.[23] Certain isothiazolone-3-one biocides have been demonstrated to exhibit a high degree of toxicity to aquatic organisms.[24] Our previous study also confirmed that Kathon biocide had a high genotoxicity in vitro and contributed to the high genotoxicity of the RO concentrate. [10] Therefore, the levels of genotoxicity and anti-estrogenic activity in the RO concentrate were caused by not only the concentration of the secondary effluent, but also some additives or chemical reactions in the mWRRO system.

Key DOM components to biotoxicity of the RO concentrate

Influence of the fluorescence characteristics

Studies have confirmed that tyrosine/tryptophan-like aromatic protein and humic/fulvic substances are important genotoxicity and anti-estrogenic activity precursors.[18,19] The correlations between the biotoxicity and the total volume of Regions I and II and the total volume of Regions III and V were studied (Figure 5). The results showed that the genotoxicity of water samples and the EEM volume of Regions I and II had a significant linear correlation ($r^2 = 0.989$, $p = .005$). Additionally, the anti-estrogenic activity and the EEM volume of Regions I and II also had a significant linear correlation ($r^2 = \ldots$)
0.985, $p = .007$). However, linear but not statistically significant correlations appeared not only between the genotoxicity and the EEM volume of Regions III and V ($r^2 = 0.828$, $p = .09$), but also between the anti-estrogenic activity and the EEM volume of Regions III and V ($r^2 = 0.878$, $p = .06$). Therefore, the fluorescence volume of Regions I and II in the EEM spectrum can be suggested as a surrogate for assessing the genotoxicity and anti-estrogenic activity per mg DOC of water samples by linear fitting.

**Influence of the size fractions**

The RO concentrates contained more contaminants and exhibited higher genotoxicity and anti-estrogenic activity; therefore, finding the key fractions that cause biotoxicity is important for selecting appropriate treatment methods. According to the results of the SEC size fraction analyses, the RO concentrates could be divided into five molecular-size fractions after UF. The distributions of the genotoxicity and anti-estrogenic activity per mg DOC of the five fractions were studied (Figure 6). The fraction containing compounds with molecular sizes from 1 to 3 kDa had both the highest genotoxicity and the highest anti-estrogenic activity per mg DOC among all of the fractions of the RO concentrate. This phenomenon was in accordance with previous reports that investigated the secondary effluent and observed that DOM with smaller molecular sizes was related to biotoxicity. [11,25]

However, DOM with MW < 3 kDa is difficult to be removed from the secondary effluent during coagulation, even at a high coagulant concentration; this difficulty leads to low removal efficiencies of compounds with anti-estrogenic activity. [25] Moreover, Bagastyo et al. [26] noted that the lower molecular-size fraction of DOM was the most difficult to eliminate in all of the investigated treatment processes (coagulation, adsorption, and advanced oxidation). Zhou et al. [27] found that ozonation-based advanced oxidation processes degrade most aromatic organics and decompose the organics with a molecular size range of 1–100 kDa into small molecular forms (< 1 kDa). Therefore, DOM fractions with MW < 3 kDa in the RO concentrate should raise more concern because of the high biotoxicity and resistance to present treatments.

**Conclusions**

The mWRRO system performs well in reducing the concentrations of inorganic salt and organic matter for wastewater reclamation. The MF filter hardly had any contribution to reduce the DOC of the secondary effluent, while the relative amount of DOM with a low MW (< 3 kDa) in the secondary effluent increased after the pretreatment process. Also, the addition of chlorine before MF promoted the formation of organics with anti-estrogenic activity. The RO permeate exhibited no genotoxicity or anti-estrogenic activity. The EEM fluorescence spectra analysis indicated that RO membrane does not concentrate everything in the secondary effluents, but changes the organic components of the secondary effluents. The RO concentrate presented higher genotoxicity and anti-estrogenic activity normalized by the DOC than the secondary effluent and MF permeate. EEM fluorescence volume for aromatic proteins could be suggested as a surrogate to assess the genotoxicity and anti-estrogenic activity of RO concentrates.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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