Assessing the Detoxication Efficiencies of Wastewater Treatment Processes Using a Battery of Bioassays/Biomarkers

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Abstract. A battery of *in vitro* bioassays, including a Neutral Red (NR) assay using MCF-7 cells for predicting cytotoxic chemicals, an ethoxy resorufin-O-deethylase (EROD) activity assay using H4IIE cells to check for dioxin-like chemicals, and a recombinant gene yeast assay for screening estrogenic chemicals, was conducted to assess the removal efficiencies of trace toxic chemicals by different treatment processes in the waste water treatment plant (WWTP). The effluents were extracted by solid phase extraction (SPE) and were fractionated into three fractions based on polarities. The battery of bioassays was performed for each fraction. In the battery, the toxicities of the effluents were described according to their modes of actions (MOA) or biomarkers and the properties of the toxic chemicals were categorized by their polarities and MOAs. The proposed procedure could be used as a tool to diagnose the toxic characteristics of the complicate mixture. The results showed that cytotoxic, dioxin-like and estrogenic chemicals could be detected in all samples. In the influent, cytotoxic and dioxin-like chemicals were mainly in polar fraction and estrogenic chemicals were in non-polar and moderate-polar fractions. The secondary treatment (active sludge) could remove a small amount of these toxicants. Among different types of advanced treatments, flocculation was good enough to remove most of the cytotoxic chemicals and a combination of flocculation, ozone oxidation, and post-biological treatment could eliminate most of the dioxin-like and estrogenic chemicals.

In recent years, with the development of industry and agriculture, many thousands of organic trace pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs) have been produced. Most of these chemicals may pose a direct or potential hazard to the aquatic biota and public health. Wastewater treatment plants (WWTPs) effluents are relevant point resources for residues of these compounds in the

aquatic environment (Clara *et al.* 2005). The parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), among others, are not feasible for the evaluation of the elimination efficiency of these groups of toxic pollutants. However, chemistry analysis, in conjunction with modern powerful chemical techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-nuclear magnetic resonance (LC-NMR), can detect increasing numbers of pollutants. But the instrumental analyses are expensive, and, in several cases, only a few percent of the organic pollutants can be identified (Betowski *et al.* 1996). It was thus considered that biological toxicity testing is a direct measure of the overall toxicity of the effluents, the intention of which is to compensate limitations arising for traditional analyses. It is for this reason that the whole effluent toxicity (WET) testing is being included by US-EPA in assessments of effluents (US-EPA 2002a).

WET testing is an integrative tool that measures the toxic effect of an effluent mixture as a whole and accounts for both uncharacterized sources of toxicity as well as toxic interactions. However, all the tests in WET are performed on a whole-organism level, where the acute or chronic effects on exposed aquatic organisms such as alga, daphnia magna, and fish are determined. Whole-organism-level tests are expensive, time- and labor-consuming, and require much laboratory space. There are great variations among the individual animal, which leads to problems with the standardization. Therefore, there is a need for a fast, reliable, reproducible, and inexpensive bioassay for the rapid screening of potential toxic pollutants in wastewater or effluents after treatment. Biomarkers based on *in vitro* bioassays offer a rapid, sensitive, and relatively inexpensive solution to some of the limitations of whole-organism-level tests. They enable estimation of the total biological activity of all compounds that act through the same mode of action present in extracts of any environmental media, and they also integrate possible interactions among chemicals (Giesy *et al.* 2002).

One of the most important toxic effects produced by toxic chemicals is the dioxin-like activity (Hilscherova *et al.* 2001). These chemicals bind to the aryl hydrocarbon receptor (AhR)

Fig. 1. Flow scheme of the advanced treatment processes and sampling locations

present in the cytosol. Their binding affinity is related to the incidence and intensity of toxic effects (Lucier *et al.* 1993, Hankinson 1995). Binding of a ligand to the AhR initiates a cascade of actions leading to enhanced transcription of AhR-regulated genes and increases in activities of AhR responsive enzymes (Zacharewski *et al.* 1995). Ethoxy resorufin-O-deethylase (EROD) is a member of the AhR-responsive enzymes. Organic compounds known to bind to the AhR include PCBs, PAHs, chlorinated dibenzodioxins, and chlorinated dibenzofurans (Hansen and Addisson 1990), which are often found together in environmental matrices. Extensive studies have suggested that H4IIE-cell EROD assay can be used to pinpoint the sources and identities of dioxin-like chemicals (Whyte *et al.* 2004).

Many recent studies have demonstrated that WWTP effluents affect the development of fish embryos and larvae (Tabata *et al.* 2001). Endocrine disruption activity in WWTP effluents has also been frequently studied (Nasu *et al.* 2001; Murk *et al.* 2002; Alatraste-Mondragon *et al.* 2003). This effect was attributed to a group of chemicals including natural products, pharmaceuticals, and industrial chemicals, which have been shown to be estrogen mimics. These substances have been associated with degenerative effects such as reduced fecundity, changes in the sex ratio, developmental degeneration in both animal and human embryos (Knörr and Braunbeck 2002), as well as an increase in breast and testis cancer in humans (Toppari *et al.*, 1996). Numerous specific testing systems have been developed for the detection of the estrogenic effects (Routledge and Sumpter 1996; Sohoni and Soto 1998; Go *et al.* 1999). Among them, recently developed recombinant yeast screen (Routledge and Sumpter, 1996) can serve as a very useful tool to rapidly and inexpensively determine the total potency of estrogenic chemicals in the mixtures of chemicals. In this yeast strain, transactivation of a receptor gene (predominantly bacterial β -galactosidase) controlled by

the respective hormone-responsive DNA elements served as proof for the functionality of the cloned protein (Routledge and Sumpter, 1996).

There are various assay methods for evaluating the cytotoxic effects of chemicals on cultured cells. The most common applications of cytotoxic testing techniques are based on cell viability. Among them, NR assay is a well-known quantitative colorimetric method (Borenfreund and Puerner, 1985). The assay is based on the uptake of neutral red dye, which accumulates in the lysosomes of uninjured cells.

Bioassay based on biomarker is promising due to its molecular and biological simplicity, easy handling, and low costs (Ramamoorthy *et al.* 1997). In addition, this assay is valuable because it is able to indicate a potential mechanism of action as well as to account for synergistic, antagonistic, and additive interactions that may occur with a complex mixture. The purpose of this report is to evaluate a battery of bioassays based on three representative biomarkers for toxicity evaluation of wastewater and effluents after treatment processes and for diagnosis of toxic characteristics of a complicated mixture.

Materials and Methods

Sample Collection and Processing

Wastewater and effluents samples were taken in May 2003 from a pilot plant in a Beijing WWTP. This pilot plant was constructed for reclamation of the wastewater after a combination of secondary and advanced treatment processes (Fig. 1). The samples include influent (A), effluent after secondary biological treatment (B), effluent from flocculation process (C), effluent from ozone oxidation process (D), and effluent from a post-biological treatment (E). Composite water samples (2L) were taken over a period of 24 h according to the protocol (US EPA, 2002a) and were processed immediately upon arrival in the laboratory. Water samples were filtered through 0.7- μ m glass fiber filters (APFF, Millipore, Bedford, MA) and extracted by solid phase extraction (SPE) using Oasis HLB cartridges (6 mL, 500 mg, Waters), which had been conditioned with 5 mL CH_2Cl_2 (HPLC Grade, Fisher), 5 mL methanol (HPLC Grade, Fisher), as well as 5 mL deionised water. The columns were then eluted three times with 5 mL CH_2Cl_2 and elutes collected were combined. This is followed by blowing it to 1 mL under a nitrogen stream (Rao *et al.* 2005).

Fractionation Based on Polarity

The extract was carefully added on a column containing 10 g silica (60–200 μ m, 60A, ultra pure, Acros Organics) and 10 g Al_2O_3 (50–200 Micron, Acros Organics) and then fractionated as described by Wu *et al.* (2002) with some modifications. Briefly, the column was eluted with 15 mL hexane (HPLC Grade, Fisher) to yield a non-polar fraction (F1), followed by 70 mL mixture of hexane/ CH_2Cl_2 (v/v=7:3, HPLC Grade, Fisher) to afford a fraction with moderate polarity (F2), and finally by methanol (30 mL, HPLC Grade, Fisher) to produce a polar fraction (F3). This separation was confirmed by using a spike recovery test and recoveries of target analytes were between 80%–105%.

The sub-fractions were evaporated to 2 mL using a rotation-evaporator, then blown to dryness with a gentle nitrogen flow. The residues were dissolved in 1 mL DMSO (ACS Grade, Amresco®) and diluted by a 0.5-fold dilution series. The DMSO solution (5 μ L) was added to 995 μ L culture medium and homogenized about 1 min in a

vortex apparatus. Thereby, maximum solvent concentration in the culture medium did not exceed 0.5%, a concentration that did not affect cell growth. Five concentration levels were chosen for dose-response relationship test. To control the effects due to DMSO, the same amount of the organic solvent was added.

Neutral Red Assay

The NR assay was conducted according to the method described by Babich and Borenfreund (1987a). Briefly, individual wells of a 96-well plate were inoculated with 0.2 mL complete medium containing 2×10^4 MCF-7 cells and incubated for 24 h. The medium was then removed and the cells were incubated with medium unamended (control) or amended with varied concentrations of test samples. After another 24 h of incubation, the medium was removed and replaced with medium containing 50 $\mu\text{g/mL}$ NR. The NR-containing medium had been preincubated overnight at 37°C and was centrifuged prior to use to remove fine precipitates of dye crystals. The microtiter plate was returned to the incubator for an additional 3 h to allow for the uptake of the dye into the lysosomes of viable, uninjured cells. Thereafter, the medium was removed and the cells were quickly washed with 0.2 mL of a fixative (1% formaldehyde-1% CaCl_2) and then 0.2 mL of a solution of 1% acetic acid-50% ethanol was added to each well to extract the dye. After an additional 10 min at room temperature and rapid agitation on a microtiter plate shaker, the plate was transferred to a microplate reader (TECAN GENios A-5002, Austria) equipped with a 535-nm filter to measure absorbance of the extracted dye.

EROD Assay

The EROD assay was performed according to the method described by Donato *et al.* (1992) and Wang *et al.* (2003). H4IIE rat hepatoma cells were grown in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum. The cells were incubated at 37°C, in a humidified 5% CO_2 incubator. For the EROD assay, cells were seeded at a density of 2×10^4 well in a 96-well plate. The cells were exposed for 72 h to medium containing different doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) standards (Cambridge Isotope Laboratories, Inc.), dimethyl sulphoxide (DMSO solvent control, Sigma, St. Louis, MO), or the test samples. Thereafter, the medium was removed and 100 μL of fresh medium containing 8 mM 7-ethoxyresorufin (Sigma) and 10 mM dicumarol (Sigma) was added for an additional 60 min of incubation at 37°C. Next, the medium was transferred to a new 96-well plate and mixed with 130 μL of absolute ethanol. Resorufin-associated fluorescence was measured with the excitation/emission wavelength of 535/595 nm (TECAN GENios A-5002, Austria). Thereafter, the total protein content was measured as described by Bradford (1976). Data obtained from EROD assays were normalized to total cellular protein. The biological TCDD-EQ values were calculated according to Hanberg *et al.* (1991) comparing the inducing of enzyme activity by environmental sample extracts with the series concentration of TCDD standard. The dose-response curves (Poch *et al.* 1995) for the induction of EROD activity were constructed by software ALLFIT. Dioxin-like potentials detected in the samples were standardized against 2,3,7,8-TCDD and expressed as TCDD equivalents (TCDD-EQ) in pg TCDD/L .

Yeast Assay

Recombinant yeast (*Saccharomyces cerevisiae*) cells were kindly provided by J.P. Sumpter from Brunel University, Uxbridge, UK. The

yeast assay was carried out as described by Routledge and Sumpter (1996) and Wu *et al.* (2002) with some modifications.

Selective medium (SC medium) was prepared following the procedures of Gaido *et al.* (1997) and Routledge and Sumpter (1996). All chemicals have a purity of >99.9 and none was re-purified prior to use. The yeast strain was grown at 30°C, 130 rpm overnight, in Erlenmeyer flasks using CuSO_4 supplemented (5×10^{-8} M) SC medium. In performing the assay, exponentially growing overnight cultures were diluted with SC medium to an $\text{OD}_{600\text{nm}}$ of 0.25.

All the samples were determined in a minimum of triplicates. Each triplicate included a positive control (17 β -estradiol) and a negative control (DMSO). The effects of estrogenic compounds were standardized against 17- β -estradiol (E_2). Each test extract was diluted in a 1:2 series in DMSO for a total of 6 concentrations. Five microliters of serial dilutions of test samples were combined with 995 μL of medium containing 5×10^3 yeast cells/mL resulting in a test culture in which the volume of DMSO did not exceed 0.5% of the total volume. Two hundred microliters of the test cultures were transferred into each well of the 96-well plate and incubated at 30°C with vigorous orbital shaking (130 rpm) on a titer plate shaker (Heidolph TITRAMAX 1000, Germany) for 2 h, then the cell density of the culture was measured at 600-nm wavelength (TECAN GENios A-5002, Austria).

A 50- μL test culture was transferred to a new 96-well plate and after addition of 120 μL of Z-buffer and 20 μL chloroform, the assays were carefully mixed (vortex 25 s) and preincubated for 5 min at 30°C. The enzyme reaction was started by adding 40 μL o-NPG (13.3 mM, dissolved in Z-buffer). The assays were incubated at 30°C on a titer plate shaker. The reactions were terminated by the addition of 100 μL Na_2CO_3 (1 M). For E_2 , the incubated time lasted for 20 min and for the samples it lasted for 60 min. After centrifugation at 12,000g for 15 min (Sigma Laborzentrifugen 2K15, Germany), 200 μL of the supernatant was transferred into a new 96-well plate and the $\text{OD}_{420\text{nm}}$ was determined. The β -galactosidase activity was calculated according to the following equations: $u = C_S/t \cdot V \cdot D \cdot \text{OD}_S$ and $C_S = 10^{-6}(A_S - A_B)/\epsilon \cdot d$ where u : β -galactosidase activity; t : incubation duration of the enzyme reaction; V : Volume of the test culture; D : dilution factor; OD_S : OD_{600} of test culture; A_S : OD_{420} of the enzyme reaction supernatant of the sample; A_B : OD_{420} of the enzyme reaction supernatant of the blank; ϵ : ϵ for o-nitrophenol in the enzyme assay reaction mix; and d : diameter of the cuvette (Routledge and Sumpter, 1996; Gaido *et al.* 1997). The estradiol equivalents (EEQ) values were calculated as described by Wu *et al.* (2002), comparing the estrogenic activity of the sample extracts with the series concentrations of E_2 standard.

In all the bioassays, the procedural blank, which had been subjected to SPE and fractionation, was also run alongside the samples to monitor for a false-positive result.

Results and Discussion

Cytotoxic Chemicals

Acute toxicities of the three fractions in the organic extracts from the effluents treated by different treatment processes were evaluated by using an NR assay. According to our fractionation protocol, F1 extract was mainly composed of chemicals such as polychlorinated biphenyls (PCBs) and alkyl-hydrocarbon. Chemicals such as polycyclic aromatic hydrocarbons (PAHs), organic chlorinated pesticides (OCPs), polychlorinated dibenzofurans (PCDFs), and dibenzo-p-dioxins (PCDDs) would be contained in F2. F3 was mainly composed of organic components such as substitution benzene, phenol,

Fig. 2. Cytotoxicity in the fractionated samples by NR assay

and organic acid (Wang *et al.* 2001a; Chul-Hwan Koh *et al.* 2002.). The results (Fig. 2) showed that the cytotoxicity was mainly contributed by the chemicals in F3. Chemicals in the other two fractions showed no obvious acute toxic effects. Among different samples, chemicals in the influent (A) induced a strong cytotoxicity response in which the cell viability was no more than 10%. In the effluent from secondary treatment (B), the cell viability rose up to more than 40%, indicating the biodegradability of some polar chemicals. The flocculation (C) removed the cytotoxicity completely and there were no toxic manifestations in effluents from the following treatments, including ozone oxidation and post-biological treatment.

Several of cytotoxicity assays have been employed for the evaluation of environment pollutants (Fent and Hunn 1996, Putnam *et al.* 2002). They have a different mechanistic basis, and thus may give some hints as to the biochemical mechanism of toxic action. The NR assay, which is one of the most commonly used cytotoxicity assays, is based on the accumulation of Neutral Red in lysosomes of viable cells (Borenfreund and Puerner 1985). Compounds causing membrane damage inhibit the accumulation of this dye, and cells lose their red color. The NR assay has been widely used in the evaluation of environmental pollutants (Babich and Borenfreund 1987b; Fent 1996) and environmental samples (Dayeha *et al.* 2002). Many compounds, including methyl mercury, chlorophenols, nitrotoluenes, chlorotoluenes, nitrophenols, methylphenols, phenols, toluenes, nonylphenol, and octylphenol, etc., have been found to be cytotoxic in the NR assay (Babich and Borenfreund 1987b). The rank order of cytotoxicity of these compounds depends on the position and the number of the substitute groups, which display different modes of toxic action. For example, the higher substituted chloro- and nitrophenols are uncouplers of oxidative phosphorylation, whereas lower substituted phenols act via narcotic-type mechanisms, 4-nonylphenol and 4-octylphenol, which primarily act on cell membranes at high concentrations and bind to the estrogen receptor at low concentrations (Fent 1996). Organotin compounds act both as inhibitors of oxidative phosphorylation and as membrane toxicants (Fent, 1996). The cytotoxicity of all these pollutants with different action modes could be clearly detected by NR assay and was found to have a good correlation with their *in vivo* waterborne acute toxicity (Babich and Borenfreund 1987b; Fent 1996). Numerous cell

viability measurements by NR assay after exposure to industrial and municipal wastewaters (Gagné and Blaise 1998) showed that comparison of data from the NR assay with those for toxic potential to rainbow trout reveal a significant relationship ($r = 0.975$, $p < 0.001$). In our study, it was found that the cytotoxicity was dominantly produced in a polar fraction, which usually has low lipophilicity. However, positive correlation between cytotoxicity and lipophilicity of substituted phenolics and chlorinated toluenes in the NR assay was found in some other studies (Babich and Borenfreund 1987b, Fent 1996). Our observation could be explained by the complexity of the environmental samples, in which thousands of pollutants with different properties are included. The integrate effects could not be predicted and explained simply by the lipophilicity as for a single compound. Therefore, in this study, in combination with sample fractionation, the NR assay could reflect the fact that most of cytotoxic chemicals in the investigated wastewater were of high polarity, and some of them could be removed by the secondary treatment and most of them by flocculation.

Dioxin-Like Chemicals

The dioxin-like chemicals were detected in all three fractions (Fig. 3). The results showed that the total TCDD-EQ of dioxin-like chemicals in the influent and the effluents was lower than 14 pg TCDD/L. This value was even far below the Drinking Water Maximum Contaminant Level (MCL value) (30 pg TCDD /L), recommended by the US EPA (2002b). The variations of TCDD-EQ in different fractions and/or in different treatment processes could be observed clearly by this biomarker. In the influent, the highest effect was produced by F3 extracts, while F1 contributed less than F2, the least to the total effects. After the secondary treatment, effects elicited by F2 and F3 extracts were raised up slightly, whereas the F1-produced effect decreased. This result suggested that some non-polar chemicals were transformed into polar and moderate polar intermediate during the secondary treatment process. It was also possible that some non-polar chemicals were eliminated by precipitation during this treatment process. Flocculation eliminated 97% F3-produced effect and 48% F2-produced effect, indicating the high efficiency of flocculation to remove polar and moderate-polar dioxin-like chemicals.

Fig. 3. Dioxin-like effects in the fractionated samples by EROD assay

Fig. 4. Estrogenic effects in the fractionated samples by Yeast assay

The F3-produced effect became higher after ozone oxidation, suggesting the formation of more polar dioxin-like chemicals, which are easier to degrade in the post-biological treatment process.

It is well known that dioxin-like chemicals include dioxins, furans, polychlorinated naphthalenes (PCNs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs), which are ubiquitous contaminants in environmental matrices, including biota, sediment, air, surface waters, and municipal and industrial effluents (Dorr *et al.* 1996; Falandysz *et al.* 1996; Harner and Bidleman, 1997; Kannan *et al.* 1998; Villeneuve *et al.* 2000). Most of these compounds are of low polarity and, therefore, are not expected to be in the polar fraction (F3). The unexpected levels of F3 dioxin-like activity possibly suggested unidentified, relatively polar, AhR agonists (Khim *et al.* 1999a,b, Brack *et al.* 2003). Another possible explanation of our observations is that the test was performed for water only and most of the hydrophobic chemical should be concentrated in the sludge or suspended matter, which was removed from the sedimentation processes. Also, dioxin-like chemicals are found in the environment as complex mixtures of both AhR-active and AhR-inactive congeners, and interactions between active and inactive congeners may complicate the effects on *in vitro* bioassay responses (Sanderson *et al.* 1996). It was illustrated that the EROD assay may underestimate the potential toxicity of chemicals, such as PAHs and

other AhR ligands that are readily metabolized (Bols *et al.* 1997; Whyte *et al.* 2004). Large molecular weight hydrophobic PAHs can be AhR active, but they are easily metabolized or transformed in part due to their lack of halogenations (Nebert *et al.*, 2000). Some of the polar chemicals, such as nitro-aromatic hydrocarbons, could also induce dioxin-like effects in EROD assay (Wang *et al.* 2001b). After treated by the flocculation, dioxin-like chemicals were largely removed and levels in effluents of ozone oxidation and post biological treatment were 2–3 pg TCDD-TEQ/L. Therefore, it could be concluded that dioxin-like chemicals in the soluble phase of wastewaters should be, at least in this case study, negligible and control of this type of chemicals could be done by efficient precipitation.

Estrogenic Chemicals

In the influent, the 17 β -estradiol equivalent (EEQ) of estrogenic chemicals was 163.4 pg EEQ /L (Fig. 4). The value is much lower than those of some WWTPs effluents in other countries (Cargouët *et al.* 2003; Pawlowski *et al.* 2004; Svenson *et al.* 2003). It can be seen that most of the estrogenic chemicals in the influent were non-polar or moderate polar. Sewage effluent has been shown to contain synthetic chemicals including nonylphenols, phthalic esters, PCBs, dioxins,

and phytoestrogens as well as synthetic estrogens from a contraceptive pill that are able to disrupt the endocrine system (Fawell *et al.* 2001; Belfroid *et al.* 1999). Desbrow *et al.* (1998) revealed that estrogenic activity in some rivers in the United Kingdom is caused by the presence of 17 β -estradiol and estrone, as well as the synthetic birth control contraceptive 17 α -ethinylestradiol. 17 β -estradiol, estrone, and 17 α -ethinylestradiol had been detected in effluents of WWTPs in Britain, Israel, and Germany. Most of these compounds are inactive after metabolizing in the body. However, microorganisms may hydrolyze sulfate and glucuronide conjugate of excreted estrogenic steroids in treated effluents, and most probably also in sewage prior to treatment (Baronti *et al.* 2000). Although these conjugates are not active estrogens, hydrolysis transforms them into active products that may exert their effect. The F-1 and F-3 produced effects could be eliminated to a very low level by the secondary biological treatment process, while the F-2 produced effect remained at the same level after this treatment process.

The flocculation process could eliminate more than 75% of the F-2 produced effect and the total effect produced by F-3. This is quite similar to the high removal efficiency of polar and moderate-polar dioxin-like chemicals in the flocculation treatment we observed in the EROD assay. However, the effect of F-1 was slightly greater after flocculation. This is also in accordance with the result we obtained in the EROD assay. In our previous study (Ma *et al.* 2001), we found in the Ames test that flocculation treatment could induce mutagenicity, which is most probably caused by chemicals with low polarity (Wang and Liu 1999). All of these results proved the fact that some non-polar chemicals with low molecular weight may be released from the large-molecule organic substances, such as humic substances, during flocculation treatment, thereby exerting toxic effects (Wang and Liu 1999).

Although ozone oxidation could remove the F1-produced estrogenic effect to a very low level, the F2-produced effect increased greatly. The increase of polarity of estrogenic chemicals by ozone oxidation might be due to the induced oxygen; thus, many chemicals are transformed into more biodegradable species in this process (Ling and Zhang 2002). Ozone oxidation followed by the biological treatment could completely eliminate the F2-produced effects, and in the final effluent 92% of effects were eliminated. Therefore, the combination of flocculation, ozone oxidation, and microbiological treatment is the most efficient way to eliminate the estrogenic effects in the influent of Gaobeidian WWTP.

Many studies have been performed on the removal of estrogenic chemicals in the sewage treatment processes. High rates of removal of natural and synthetic estrogens in biological sewage treatment plants were found in Brazil and Germany (Ternes *et al.* 1999). Sewage treatment processes in Italy could remove 61–95% of E1, E2, and EE2. However, some estrogenic chemicals could not be eliminated by the secondary treatment. It was reported that certain natural and synthetic hormones and alkyl-phenolic surfactants could not be removed during the biological treatment process and their estrogenic effects were even increased or activated (Nasu *et al.* 2001). In comparison, we can conclude that the removal efficiency of estrogenic chemicals from secondary treatment process depends on the chemical characteristics of the sewage. In our case, the mixture of both domestic and industrial wastewater in

the influent to the WWTP complicates the situation and the integrative treatment system is efficient for removal of these chemicals.

Conclusions

By using a battery of bioassays, levels of cytotoxic, dioxin-like, and estrogenic chemicals in influent of WWTP and effluents of different treatment processes could be evaluated. The combination of fractionation and bioassays based on biomarkers is especially useful to identify the properties of these chemicals and elucidate the mechanism of each treatment process for each category of chemical. The proposed procedure could be used to assess the technique feasibility when reclamations of the wastewater are of ecological and health concerns. For example, it could be used in the screening stage (Tier 1) of WET and be used as a tool of diagnosis in the toxic identification and evaluation procedure (TIEs).

The secondary treatment in WWTP is efficient to remove the acute toxic chemicals as well as non-polar dioxin-like and estrogenic chemicals. The flocculation, as an advanced treatment technology, could eliminate most of the polar and non-polar dioxin-like and estrogenic chemicals. More polar or moderate polar dioxin-like and estrogenic chemicals were formed by ozone oxidation. However, it leads to the chemicals becoming more biodegradable and thus much easier to remove by following the biological treatment.

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