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AOX contamination status and genotoxicity of AOX-bearing pharmaceutical wastewater

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ABSTRACT

Adsorbable organic halogens (AOX) are a general indicator for the total amount of compounds containing organically bonded halogens. AOX concentrations and components were investigated along the wastewater treatment process in four large-scale pharmaceutical factories of China, and genotoxicity based on the SOS/umu test was also evaluated. The results showed that AOX concentrations in wastewater of four factories ranged from 4.6 to 619.4 mg/L, which were high but greatly different owing to differences in the raw materials and products. The wastewater treatment process removed 50.0%–89.9% of AOX, leaving 1.3–302.5 mg/L AOX in the effluents. Genotoxicity levels ranged between 2.1 and 68.0 μ g 4-NQO/L in the raw wastewater and decreased to 1.2–41.2 μ g 4-NQO/L in the effluents of the wastewater treatment plants (WWTPs). One of the main products of factory I, ciprofloxacin, was identified as the predominant contributor to its genotoxicity. However, for the other three factories, no significant relationship was observed between genotoxicity and detected AOX compounds.

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Introduction

Adsorbable organic halogens (AOX) are a general indicator for the total amount of organically bonded halogens, which comprises many groups of compounds containing at least one halogen atom in a molecule (Kaczmarczyk and Niemirycz, 2005). AOX includes chemicals of different structures and toxicological profiles, many of which persist in the environment, accumulate in the food chain and pose serious adverse health and environment risks (Müller, 2003). For example, 56% of the priority pollutants identified by the US EPA as well as 23 kinds of persistent organic pollutants (POPs) selected by the Stockholm convention are AOX compounds. Due to the endocrine-disrupting and toxic nature of many halogenated compounds (Dann and Hontela, 2011), the presence of AOX in wastewater has raised concern. Thus, as a sum parameter of halogenated compounds, AOX has been strictly regulated by

integrated wastewater discharge standards in many countries, including China (Luyten et al., 2013).

Appreciable AOX was detected in municipal wastewater (Clara et al., 2012), landfill leachate (D. Goi, 2009) and hospital wastewater (Kuemmerer et al., 1998), as well as in many industrial wastewaters, such as those from the pulp paper industry (Kumar et al., 2013), dying and textile industry (Alves et al., 2014), and other industries (Luyten et al., 2013; Van Aken et al., 2013). China has limited the AOX concentration in the national discharge standards of those industrial effluents. However, AOX in pharmaceutical wastewater has rarely been studied and is not restricted in the current national discharge standard, although organic halogens are widely used in the pharmaceutical industry (Priya and Philip, 2013). Furthermore, halogen bonding is extremely important for blocking metabolism and enhancing the biological activity of pharmaceuticals (Wilcken et al., 2013), thus many drugs are organic

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halogen compounds as well. The limited recovery rate of these materials and products is expected to result in high concentrations of AOX in pharmaceutical wastewater.

Short-term genotoxicity tests are useful for monitoring the genotoxicity of organic halides and unidentified chemicals (Le Curieux et al., 1996). The SOS/umu test is one of the most frequently applied techniques to assess the presence of genotoxic contaminants in wastewater (Krishnamurthi et al., 2008). It was demonstrated that genotoxicity decreased as AOX was removed from industrial wastewater (Chaparro and Pires, 2011). Nevertheless, the genotoxicity of AOX compounds in pharmaceutical wastewater has not yet been investigated in detail.

The Chinese pharmaceutical industry has developed rapidly in recent decades. Four large-scale pharmaceutical factories (named I, II, III and IV) located in Zhejiang Province of China were selected to examine the AOX contamination status and genotoxicity of their wastewaters. Factory I is the world's largest production enterprise for ciprofloxacin. Factory II is the largest tetrachlorophthalic anhydride producer in the world. Factories III and IV are both the main manufacturers of their core products (vitamins for III, chemically active pharmaceutical ingredients for IV) in China.

1. Materials and methods

1.1. Wastewater and sludge samples

Every factory has its own wastewater treatment plant (WWTP) to pretreat wastewater before discharging into the comprehensive WWTP of the industrial park. Generally, wastewater from

different workshops is mixed together in the conditioning tank to homogenize the wastewater quality and then pumped into the biological treatment process. The hydrolytic acidification process, anoxic process, anaerobic process, aerobic process, cyclic activated sludge system (CASS) and settling are included in the biological treatment processes of these 4 WWTPs (Fig. 1).

Water samples were withdrawn along the biotreatment process (Fig. 1). According to the regulations of these factories, it was forbidden to withdraw the wastewater of each workshop and settling tank. Thus, samples after the conditioning tank, which were a mixture of different workshop wastewaters in a factory, and before the settling tank, were considered as the influent (raw wastewater of factory) and effluent of WWTPs, respectively. The hydraulic retention time was not considered during sample collection. The wastewater quality was relatively stable since the production processes of all 4 factories were in full working order during January, March and July, and the wastewaters were further homogenized in the conditioning tank. Besides, the wastewater was completely mixed in each separate treatment unit rather than going through the unit in a plug flow pattern. Thus, it was considered that the collected effluent corresponded to the respective influent.

Samples were withdrawn in January (factories I and III), March (factories I, II and III), and June (I, II, III and IV) of 2014, during which all four factories' production processes and wastewater treatment processes were in full working order. For each sample site, 2 L wastewater was taken into glass or polyethylene bottles. The bottles were pre-washed first with tap water and deionized water in sequence, then acid-washed with 2 mol/L nitric acid (Shomar, 2007). Samples were transported to the laboratory for AOX determination and prepared for other determinations.

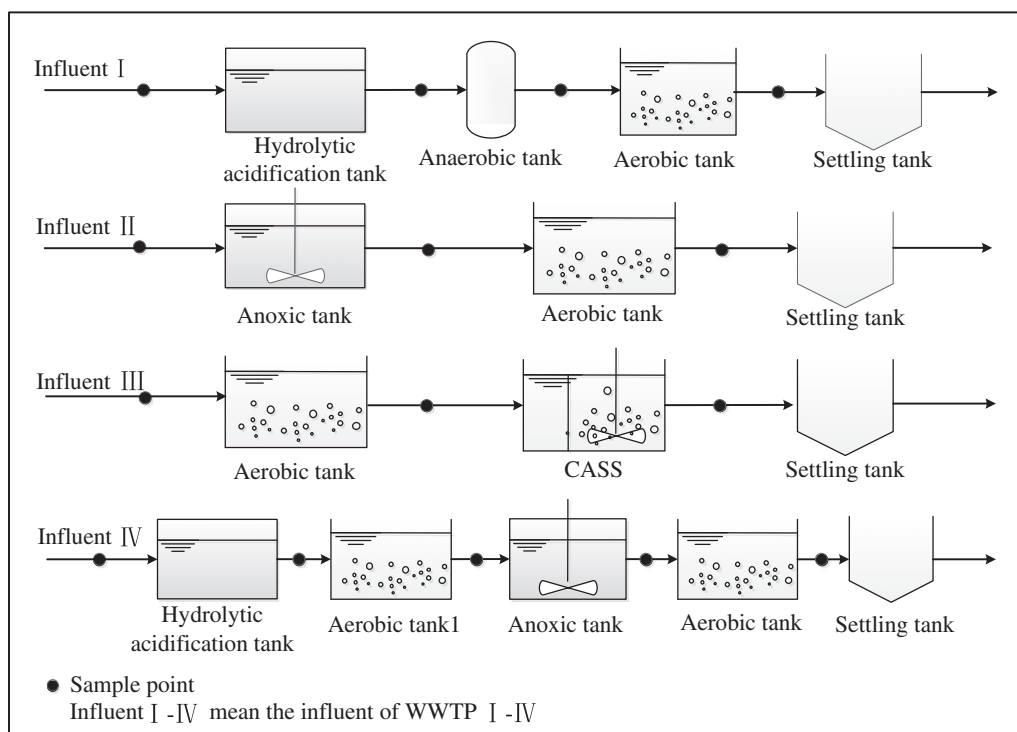


Fig. 1 – Sample points and the biological treatment process of 4 wastewater treatment plants (WWTPs). CASS: cyclic activated sludge system.

The activated sludge samples were collected from the same sites as the wastewater. Twenty liters of the mixture from those tanks containing about 3000 mg/L sludge was taken and settled for 30 min to concentrate the sludge, then the supernatant was discarded; this was repeated 5 times. Sludge samples were freeze-dried at -50°C in the laboratory, then ground, homogenized and sieved to 100 mesh, and stored in a desiccator for determination.

1.2. Determination of AOX

AOX was determined by an AOX/TOX analyzer multi X 2500 (Jena, Germany). Briefly, each wastewater sample (100 mL) was put into an Erlenmeyer flask and acidified to $\text{pH} < 2$ with 16 mol/L nitric acid. Then 5 mL $\text{NaNO}_3\text{--HNO}_3$ solution (see below) and 50 mg activated carbon powder (Jena, Germany) were successively added into the Erlenmeyer flask. The Erlenmeyer flasks containing 100 mL samples, 5 mL $\text{NaNO}_3\text{--HNO}_3$ solution, and 50 mg activated carbon powder were shaken for 1 hr at 180 r/min. After being shaken, the mixture in the Erlenmeyer flasks was filtered with an automatic filtration unit equipped with quartz columns (Jena, Germany). The 50 mg activated carbon powder was thereby retained in the quartz columns. Then, the quartz columns were put into the analyzer to determine AOX. For quality control, three external points were applied for each time analysis.

For determination of the AOX concentration in sludge, 10 mg freeze-dried sludge, 10 mL $\text{NaNO}_3\text{--HNO}_3$ solution and 20 mg activated carbon were used instead of the 100 mL wastewater, 5 mL $\text{NaNO}_3\text{--HNO}_3$ solution and 50 mg activated carbon mentioned above.

The $\text{NaNO}_3\text{--HNO}_3$ solution was prepared as follows: 17 g NaNO_3 was dissolved in 500 mL deionized water, then 25 mL HNO_3 was added and finally the system was diluted to 1000 mL with deionized water.

1.3. Genotoxicity assay

Wastewater samples (200 mL) for genotoxicity assay were prepared using solid phase extraction (SPE) with Oasis HLB cartridges (6 mL/500 mg, Waters Corporation, USA). The cartridges were conditioned with 5 mL methanol and 5 mL deionized water. The flow rate used for extraction of organics was about 6 mL/min. The cartridges were kept under vacuum until they were completely dried. Organics retained on the cartridge were eluted with 5 mL dichloromethane, 5 mL hexane and 8 mL acetone respectively and completely dried under a nitrogen flow as previously reported (Wu et al., 2010). The residues were then dissolved in 1 mL Dimethyl sulfoxide (DMSO) (99%, Sigma-Aldrich, USA), then stored at -20°C .

The genotoxicity of the wastewater samples was evaluated with the SOS/umu test based on *salmonella typhimurium* TA1535/Psk1002 without S9 activation according to a previous report (Ye et al., 2014). In this assay, the β -galactosidase induced by genotoxic chemicals was measured to monitor overall genotoxicity. A DMSO solution of 4-nitroquinoline-N-oxide (4-NQO) was used as the positive control. The genotoxicity of the samples was standardized to an equivalent 4-NQO concentration. Each sample was analyzed in triplicate, and finally the average value was recorded.

1.4. AOX compounds analysis

Samples were prepared with a modified method based on a previous report (Liu et al., 2014). Wastewater samples (200 mL) were filtered through 0.7 μm membranes (Waterman, USA) and extracted with Oasis HLB cartridges (6 mL/500 mg, Waters Corporation, USA) on a vacuum extraction manifold. The cartridges were conditioned with 5 mL methanol and 5 mL deionized water in sequence. Organics were extracted at a flow rate of approximately 6 mL/min and eluted with 5 mL dichloromethane and hexane (99%, Sigma, USA), respectively. Samples were reconstituted to 1 mL in dichloromethane and hexane, respectively.

For the detection of volatile organic halogens, the 120 mL serum bottles were completely filled with wastewater and sealed with Teflon-coated butyl rubber stoppers, and crimped with an aluminum crimp cap, immediately after the wastewater samples were withdrawn in the WWTPs. Before analysis, 30 mL of wastewater was drawn out, leaving 90 mL wastewater in the serum bottles. Then the bottles were heated to 70°C for 30 min in a water bath. Then 500 μL of the head gas was injected into a gas chromatograph.

Identification of AOX compounds in wastewater was carried out using an Agilent 7890 A gas chromatograph (GC) equipped with a unipolar 5973 mass selective detector (MS) and HP-5MS (5% Phenyl Methyl Siloxane, 30 m, 250 μm , 0.25 mm) capillary column with nitrogen as a carrier gas (24 mL/min). The injector temperature was 280°C . A one microliter sample was injected in the GC, which was operated with a split rate of 20:1. The initial oven temperature was held at 50°C for 2 min and increased to 110°C at $50^{\circ}\text{C}/\text{min}$, which was held for 2.5 min, then increased to 320°C at $10^{\circ}\text{C}/\text{min}$, which was held for 1 min. The gas chromatography mass spectrometry (GC-MS) analysis was carried out with electron-impact ionization at 1200 V, and the spectra were recorded in the interval 45–500 amu. Identifications were carried out with the aid of the NIST database library.

2. Results

2.1. AOX contamination status

Average values of AOX concentrations after each process are shown in Fig. 2, and detailed data are provided in Appendix A Fig. S1 of the supplementary material. The AOX concentration varied dramatically depending on the wastewater. The highest AOX concentration was detected in the influent of WWTP II, which was about 135 times higher than that of factory IV. The wastewater treatment process removed 50.0%–88.9% of AOX, leaving 1.3–302.5 mg/L AOX in the effluent. Furthermore, relatively high AOX concentrations were detected in the activated sludge. Higher AOX concentrations were detected not only in wastewater of factory I and II but also in sludge of these factories compared with that of the other two factories. This implied that a high AOX concentration in the liquid phase could be an indicator of a high concentration in sludge due to adsorption. On the other hand, there was a risk that the sludge accumulated large amounts of AOX when the WWTP suffered from high AOX wastewater.

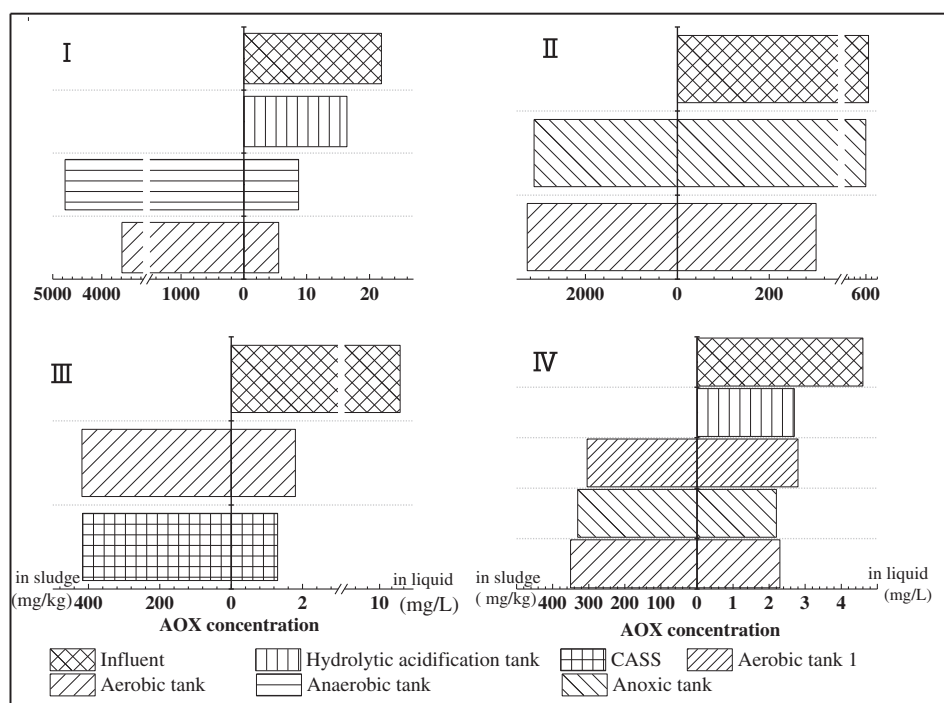


Fig. 2 – AOX concentration of samples of the investigated pharmaceutical factories.

2.2. Identification of AOX compounds

AOX components of July samples were identified by GC–MS (Table 1), and the GC–MS data are provided in detail in Appendix A Figs. S2–S25. The AOX compounds strongly depended on the materials used and the products produced

by these factories. 2, 4-dichloro-5-fluoroacetophenone was the main raw material of factory I, and was detected in all treatment units of its WWTP. Similarly, chlorobenzoic acids and 4, 5, 6, 7-tetrachloro-1, 3-isobenzofurandione were the raw materials and the main product of factory II. The wastewater of factory III was the only one in which a volatile AOX,

Table 1 – Genotoxicity of AOX compounds detected in pharmaceutical wastewater.

AOX compounds	Detected in wastewater of factory	Genotoxicity	References
1, 3-Dichloro-4-fluorobenzene	I	N	This study
3-Chloro-4-fluoroacetophenone	I	N	This study
1, 2, 3-Trichlorobenzene	I	N	This study
2, 4-Dichloro-5-fluoroacetophenone	I	N	This study
1, 2, 4, 5-Tetrachlorobenzene	I II III IV	N	This study
2, 6-Dichloro-4-(1, 1-imethylethyl)-phenol	I	–	
1-(5-Chloro-2-hydroxyphenyl)-ethanone	I	N	This study
3, 4-Dichloro-2, 5-furandione	II	N	This study
2-Chlorobenzoic acid	II	N	This study
2, 3, 6-Trichlorophenol	II	N	(Reifferscheid and Hell, 1996)
5, 6-Dichloro-1, 3-isobenzofurandione	II	N	This study
2, 3, 6-Trichlorobenzoic acid	II	–	
2, 3, 4, 5-Tetrachlorobenzoic acid	II	–	
4, 5, 6, 7-Tetrachloro-1,3-isobenzofurandione	II	N	This study
Dichloromethane	III	P	(Reifferscheid and Hell, 1996)
1, 2, 4-Trichlorobenzene	III	N	This study
(2-Chloroethenyl) benzene	IV	–	
3-Chlorobenzaldehyde	IV	N	This study
2-Trichloromethyl-1-oxaspiro [4, 5]dec-2-en-4-one	IV	–	

N means the result of genotoxicity test was negative.

P means the result of genotoxicity test was positive.

– means the pure compounds were unavailable.

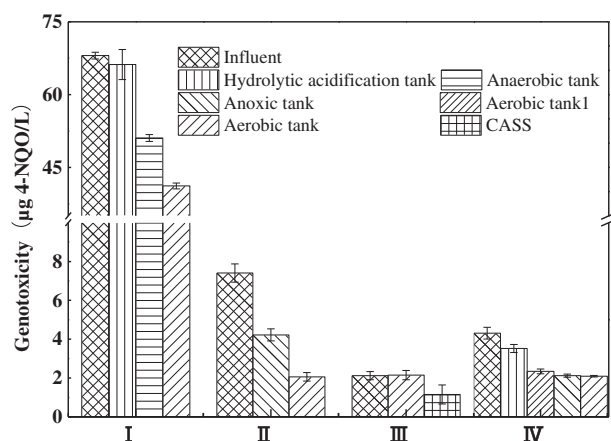


Fig. 3 – Variation of genotoxicity along the biological treatment process of WWTPs.

dichloromethane, was detected. The dichloromethane was used in factory III's process for producing vitamin E. Few AOX compounds were detected in the wastewater of factory IV, which related to its low AOX concentration. It is noteworthy that chlorobenzenes were detected in all four wastewaters.

2.3. Genotoxicity of the pharmaceutical wastewater

The genotoxicity was evaluated based on the SOS/umu test (Fig. 3). The influent of the WWTP of factory I (hereafter shown as WWTP I) exerted the highest genotoxicity, which was 9–32 times higher than that of the other 3 influents. The tendency of detoxification along the treatment process was observed in all 4 WWTPs. The anaerobic process removed 25% and 43% of genotoxicity from the influent of WWTPs I and II, respectively. The aerobic process of the WWTPs of factory I, II and IV removed 19.3%, 51.1%, and 33.5% of genotoxicity, respectively, while its effect was negligible in WWTP III. All effluents of the 4 WWTPs were genotoxic, among which the effluent of WWTP I exerted about 19 times higher genotoxicity than that of the other factories.

In addition, the genotoxicity significantly correlated with AOX values in the wastewaters of factories I and II, which was implied by Pearson's correlations ($r = 0.955$ and 0.863 ,

respectively, $p < 0.05$). However, they were insignificantly correlated with each other in the wastewaters of factory III and IV ($r = 0.870$, $p = 0.055$; $r = 0.421$, $p = 0.226$, respectively), which contained less AOX.

3. Discussion

3.1. AOX concentration in different wastewaters

Various AOX concentrations were reported in different wastewaters, such as dying industry wastewater, pulp paper industry wastewater, hospital wastewater and landfill leachate, by previous studies, as shown in Table 2.

AOX concentrations in pharmaceutical wastewater were similar to that of pulp paper industry wastewater, except for the wastewater of factory II, which contained extremely high AOX, and were higher than those of other industrial wastewaters.

Organic chemicals may be adsorbed onto sludge (Harrison et al., 2006). AOX concentrations in the activated sludge of WWTPs treating pharmaceutical wastewater were much higher than other previously reported values. However, very few countries set rules limiting the concentration of any organic chemicals in sewage sludge, and the AOX concentration remains unregulated as well (Harrison et al., 2006). China and Germany both limited AOX to 500 mg/kg in sludge for use in agriculture (Laternus et al., 2007), which can be considered as an indirect limit due to the lack of AOX limitations for sewage sludge. Sludge from the WWTPs of factories I and II significantly exceeded this limit, and the other two came close to the limitation.

3.2. Variation of AOX compounds along the treatment process

AOX components strongly depended on the materials and products of factories. Halogenated materials and products both increased AOX concentrations in industrial wastewater (Emmanuel et al., 2004; Hofl et al., 1997). Additionally, the biological transformation of biotreatment complicated the AOX composition in the effluent of WWTPs (van Pée and Unversucht, 2003).

The compound 2, 4-dichloro-5-fluoroacetophenone was the main material of factory I. It was probable that other AOX

Table 2 – AOX concentrations in different environments.

	AOX	Source of wastewater/sludge	References
Wastewater samples (mg/L)	0.4–1.6	Dying industry	(Gunasekaran and Kanmani, 2014; Ramjaun et al., 2011)
	2.8–83.0	Pulp paper industry	(Dorica, 1992; Muhamad et al., 2012; Ugurlu and Karaoglu, 2009)
	2.2–4.4	Landfill leachate	(Goi et al., 2009; Noma et al., 2001)
	0.7–10.0	Hospital wastewater	(Emmanuel et al., 2004; Gartiser et al., 1996; Kuemmerer et al., 1998)
	0.02–0.3	Municipal wastewater	(Jancewicz et al., 2011)
Sludge samples (mg/kg)	3.0, 5.0	Pharmaceutical industry	(Hofl et al., 1997)
	4.6–619.4	Pharmaceutical industry	This study
	580.0–733.0	Municipal wastewater treatment plant	(Benabdallah El-Hadj et al., 2007)
	150.0–493.0	Pulp paper wastewater treatment plant	(Welker and Schmitt, 1997)
	500.0	Industry park wastewater treatment plant	(Shomar, 2007)
	304.3–4753.7	Pharmaceutical wastewater treatment plant	This study

compounds detected in subsequent tanks were converted from 2, 4-dichloro-5-fluoroacetophenone since those compounds were not detected in the influent, and they had a molecular structure similar to 2, 4-dichloro-5-fluoroacetophenone. Similarly, trichlorobenzoic acids and other chloroisobenzofurandiones were converted from 4, 5, 6, 7-terachloro-1, 3-isobenzofurandione and 2, 3, 4, 5-terachloro-benzoic acid, the main product and material of factory II, in the wastewater treatment process.

Dichloromethane, which has been widely used as a solvent in the pharmaceutical industry, was detected in the wastewater of factory III (Priya and Philip, 2013). Due to the strong volatility of dichloromethane and the blow-off effect of the aeration tank, dichloromethane could not be detected in aerobic tank effluent. The AOX concentration dropped dramatically in the aeration process; however, the reduction nearly stopped in CASS. These results indicated that dichloromethane played an important role in the AOX of wastewater from factory III. Few compounds other than tetrachlorobenzene were detected in factory IV's wastewater.

Previous research found that tetrachlorobenzenes can be biodegraded to trichlorobenzenes (Langenhoff et al., 2013) and chlorophenols in oxygen-limited conditions (Vogt et al., 2004). These results explained the appearance of trichlorobenzenes and chlorophenols in the anaerobic and aerobic tank, although they were not detected in the influent of WWTP I, II and III.

3.3. Genotoxicity of pharmaceutical wastewater and AOX compounds

The SOS/umu test was used to evaluate the genotoxicity of the pharmaceutical wastewaters. The genotoxicity level of the pharmaceutical wastewaters was similar to that of municipal wastewater, while being lower than reported industrial wastewater, and higher than that of other sources (Table 3).

The genotoxicity significantly correlated with AOX values in wastewater containing high AOX, such as wastewater from factories I and II. The genotoxicity of detected AOX compounds was tested except for those chemicals that were unavailable. For factory I, all of the GC-MS detected AOX compounds were non-genotoxic (Table 1). However, high concentrations of ciprofloxacin hydrochloride were detected (using HPLC (Ding et al., 2015)) in wastewater of factory I: 5.06, 4.88, 4.00 and 3.23 mg/L in the influent, hydrolysis acidification tank, anaerobic tank, and aerobic tank, respectively. The genotoxicity of ciprofloxacin was 0.012 μg 4-NQO/ μg cip according to our test. Therefore, ciprofloxacin contributed importantly to the genotoxicity of factory I's wastewater.

The results of genotoxic testing of 4, 5, 6, 7-terachloro-1, 3-isobenzofurandione, chlorobenzoic acids, chlorobenzenes and chlorophenols were negative based on our test and previous reports (Table 1). These results indicated that the genotoxicity of wastewater of factory II was not contributed by the detected AOX compounds.

Dichloromethane is genotoxic. However, the extraction process, especially the nitrogen blow-off process during sample preparation, could completely remove the dichloromethane from samples. Therefore, even though dichloromethane was genotoxic, it was not the contributor of genotoxicity in the wastewater of factory III. The similar genotoxicity values of the influent and the aerobic tank effluent confirmed this result (Fig. 3).

Furthermore, studies have drawn different conclusions on the relationship between AOX concentration and toxicity depending on the wastewater selected. In pulp paper industry wastewater containing high AOX concentrations, a significant relationship was shown between AOX and toxicity (Chaparro and Pires, 2011). However, no significant relationship was observed between AOX and genotoxicity in chemical wastewater treated by the O_3 /activated carbon process (Gonzalez et al., 2003). In another study of chemical wastewater, AOX showed a higher Spearman coefficient with toxicity than other conventional indicators like TN and TOC; nonetheless, it was not strong enough to conclude that toxicity was sensitive to AOX (Gellert, 2000). This study showed that it was hard to conclude that AOX compounds contributed to the genotoxicity without further identification. However, they may exert other toxicity. For example, chlorophenols detected in the aerobic tank of factory II were proven to be genotoxicity negative, but they exhibit toxicity toward *Daphnia magna* and the alga *Scenedesmus obliquus* (Xing et al., 2012).

All four pharmaceutical wastewaters were polluted by AOX. The AOX concentrations and compounds of different pharmaceutical wastewaters varied dramatically due to differences in the materials and products. Additionally, AOX accumulated in the sludge of WWTP systems. Potential risks could be posed to soil and terrestrial organisms if the AOX-bearing sludge were left untreated. AOX should be monitored as a sum parameter of the pharmaceutical industry for effluent and sludge, and pretreatment should be enhanced to keep the WWTP free from persistent AOX compounds to improve effluent quality and reduce the environment contamination. However, extreme care should be taken when concluding a relationship between genotoxicity and AOX compounds.

Table 3 – Genotoxicity of different liquid.

Genotoxicity (μg 4-NQO/L)	Type of wastewater	References
0.01–0.21 (raw water)	waterworks from 5 basins of China	(Wang et al., 2011)
0.11–1.03 (finished water)		
0.258–0.451	Soluble microbial products of municipal wastewater	(Zhang et al., 2015)
0.6–0.8	Pulp and paper mill wastewater	(Huang et al., 2015)
105.0–160.0	Industrial wastewater	(Tang et al., 2013)
4.5–56.5	Municipal wastewater	(Wu et al., 2010)
		(Wang et al., 2007)
2.1–68.0	Pharmaceutical wastewater	This study

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jes.2016.04.014>.

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