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Effect of Ammonia Nitrogen and Dissolved Organic Matter Fractions on the Genotoxicity of Wastewater Effluent during Chlorine Disinfection

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Chlorine is a widely used disinfectant which prevents the spread of harmful pathogens when reusing wastewater, but harmful byproducts might be formed and cause adverse ecological and health effects. In this study, the potential effects of chlorination on the genotoxicity of different biologically treated wastewater samples were investigated using the *umu* test. For the first time, ammonia nitrogen (NH_3—N) was found to significantly influence genotoxicity during wastewater chlorination. After chlorination, the genotoxicity decreased in wastewater with a low NH_3—N concentration (<10∼20 mg/L), but it increased notably in wastewater with a high NH_3—N concentration (>10∼20 mg/L). By fractionating the DOM (dissolved organic matter) in wastewater into different fractions, it was found that the hydrophilic substances (HIS) fraction of DOM was the key fraction involved in decreasing genotoxicity during the chlorination of wastewater with a low NH_3—N concentration, while the hydrophobic acids (HOA) fraction of DOM was the key fraction involved in increasing genotoxicity during chlorination of wastewater with a high NH_3—N concentration. Furthermore, fluorescence spectroscopy analysis on different fractions indicated that some free or combined aromatic amino acids might produce highly genotoxic byproducts during the chlorination of wastewater with a high NH_3—N content, and this was then demonstrated through experiments on the chlorination of free aromatic amino acids.

Introduction

Wastewater reclamation and reuse is a viable and attractive approach to solving water shortages in many countries. In order to prevent the spread of harmful pathogens in reclaimed wastewater, chlorine disinfection has been widely used, but many researchers have reported that chlorine could react with dissolved organic matter (DOM) to produce numerous disinfection byproducts (DBPs) with genotoxic, mutagenic and/or carcinogenic activity (*1*−*3*).

For chlorination of drinking water, most researchers focused on the reaction of chlorine with natural organic matter (NOM) in the formation of typical DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs). However, compared to drinking water, there are many more types of DOM in biologically treated wastewater, including residual non- or slowly biodegradable influent substrates, degradation intermediates, end products, and soluble microbial products (*4*). On one hand, some of the DOM are toxic themselves (*4*, *5*), on the other hand, some forms of DOM react with chlorine to form toxic DBPs (*2*, *4*). Therefore, only measuring the formation of typical DBPs is not sufficient to investigate the effect of chlorination on the ecological safety of wastewater.

The application of short-term genotoxicity tests for wastewater is helpful in monitoring genotoxic DBPs and in checking for the presence of unknown substances with genotoxic properties (*6*−*7*). Although some researches have studied the effect of chlorination on wastewater with the Ames test, there are no consistent conclusions. Studies by Meier (*8*) and Crebelli (*9*) demonstrated that chlorination of secondary effluents did not play a substantial part in mutagenicity. However, Meier's (*10*) later study and Fukui's (*11*) study showed that chlorination resulted in substantial increases in mutagenicity in some wastewater. Research conducted by Nakamuro (*12*) on the Yodo river in Japan which had received the effluent of a wastewater treatment plant indicated that chlorination could reduce mutagenicity. The inconsistencies among these results suggest that chlorination can influence wastewater genotoxicity in a way which is specific to the wastewater samples used. The aim of this study was, therefore, to investigate the effect of chlorine disinfection on genotoxicity for different kinds of biologically treated wastewater and also to clarify the frequency of genotoxic effects, by using the in vitro *umu* test with the genetically modified strain TA1535/pSK1002 of *S. typhimurium* which allows to measure the induction of SOS repair response after contact with genotoxic compounds.

Materials and Methods

Wastewater Samples. Wastewater samples used in this study were collected from the effluents of different domestic wastewater treatment and reclamation plants before disinfection, in which activated sludge process (AS), anaerobic−anoxic−oxic process (AAO) and membrane bioreactor (MB) were used as the treatment methods. The samples were immediately delivered to the laboratory after sampling and filtered to eliminate any suspended solid before use.

Chlorination Treatment. Chlorine disinfection was conducted within 24 h of sampling. A series of 600 mL glass bottles with Teflon inner plugs were prepared, and each bottle was filled with about 580 mL of the sample wastewater. The pH of the samples was adjusted to 7.0 ± 0.2 with 1 M H_2SO_4 or 2 M NaOH solution, then 12.5 mL of buffer solution (34.0 g/L KH_2PO_4 and 35.5 g/L Na_2HPO_4) was added to keep the pH stable during disinfection. Different concentrations of available chlorine were added into the corresponding reaction bottles by using a NaClO solution with a concentration of 5 g available chlorine per liter. The bottles were sealed and kept in a dark isothermal chamber (20 °C) for 30 min. All of the chemical reagents used were of an analytical purity.

Water Quality Measurements. The pH value of the wastewater samples was measured in the field during the sampling process, and other parameters were measured after sampling and filtration. Concentration of chemical oxygen demand (COD) was analyzed by a closed reflux digestion and a titrimetric method. Concentration of ammonia nitrogen (NH3—N) was determined by colorimetry using the nesslerization method. Concentration of dissolved organic carbon (DOC) was detected using a TOC analyzer (model TOC-5000A, Shimadzu, Japan). UV absorbance at 254 nm (UV254) was measured with a photospectrometer (model UV-2401, Shi-

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madzu, Japan). Residual chlorine was measured by DPD colorimetric method (13).

Concentration of Wastewater Samples. Wastewater samples before and after disinfection treatments were acidified to pH 2 with 2 M H_2SO_4, and passed through resin cartridges which were filled with 1 g of CHP20P resin (Mitsubishi Chemical Corporation, Japan). The cartridges had previously been washed with 10 mL of methanol, 10 mL of acetone and 20 mL of distilled water. Five hundred milliliters of each sample was loaded on a cartridge, then dried under a flow of nitrogen and eluted with 4 mL of acetone. The eluate was reduced to a small volume by vacuum evaporation and then dried under a nitrogen flow. The dry residue was dissolved in 2 mL of dimethylsulfoxide (DMSO) and stored in the dark at −20 °C before used in the *umu* test.

***Umu* Test.** The *umu* test for genotoxicity was performed with *Salmonella typhimurium* TA1535/pSK1002 without S9 activation according to ISO 13829 (*14*). Concentrated samples were submitted to the test and carried out in triplicates. During each test, a series of 4-NQO (4-nitroquinoline-N-oxide) reference samples with different concentrations were run concurrently to obtain the dose−effect curve of 4-NQO. The genotoxicity of the concentrated samples was standardized to an equivalent 4-NQO concentration and it was then divided by concentration ratio to get the genotoxicity value of the samples before concentration.

Fractionation. Methods for isolating hydrophilic substances (HIS), hydrophobic acids (HOA), hydrophobic neutrals (HON), and hydrophobic bases (HOB) fractions of DOM are specified by Huang (*15*) and briefly described as follows: Five liters of wastewater sample passed through XAD 8 column and the column was back-flushed with 0.1 N HCl to elute HOB fraction. Then the previous effluent from the column was acidified to pH 2 and passed through the column again. The effluent was HIS fraction and the column was back-flushed with 0.1 N NaOH to elute HOA fraction. Finally, HON fraction adsorbed in the XAD 8 resin was Soxhlet-extracted with anhydrous methanol. After fractionation, the pH value of each fraction was adjusted to 7.0 ± 0.2 and then MilliQ water was added to each fraction to increase the volume to 5 liters.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a fluorescence spectrophotometer (model F-2500, Hitachi, Japan). Three-dimensional spectra were obtained by measuring the emission spectra in the range from 240 to 600 nm repeatedly, and at the excitation wavelengths from 220 to 420 nm, at 5 nm intervals in the excitation domain. Spectra were then concatenated into an excitation emission matrix (EEM). The three-dimensional plots and contour maps were produced using the OriginPro 7.5 program. All contour maps were plotted using the same scale range of fluorescence intensities and number of contours.

Results and Discussion

Characteristics of the Wastewater Samples Used. Table 1 shows the characteristics and genotoxicity of wastewater samples A∼G. Their genotoxicity ranged within equivalent of 4.5−56.5 μg of 4-NQO per liter, and there were no obvious relationships between the genotoxicity and the value of COD, NH_3−N, DOC, and UV_{254}. Since the effluents from biological wastewater treatment systems contain a variety of types of DOM (*4*), the genotoxicity was considered to derive from complex mixtures and, therefore, could not easily be estimated from the conventional water quality indices.

Effect of Chlorination on Wastewater Genotoxicity. The genotoxicity of samples A∼E after disinfection with differing chlorine dosages is shown in Figure 1. It can be seen that with chlorine dosage increasing, the genotoxicity of samples A, D, and E decreased, while the genotoxicity of sample B increased, and finally, the genotoxicity of sample C did not change that much. After analyzing their water quality data (Table 1), it is found that the NH_3−N concentration of samples A, D, and E was much lower than that of sample B, and that the NH_3−N concentration of sample C was midway between them. The NH_3−N concentration was, therefore, hypothesized to be the key parameter resulting in the differing patterns of genotoxic change during chlorination.

To investigate the effects of ammonia nitrogen, NH_4Cl was added to samples D and E which had low NH_3−N concentrations originally. After samples D and E with altered NH_3−N concentrations were disinfected with 10 and 30 mg/L of chlorine for 30 min, both the genotoxicity and the total residual chlorine were measured (see Figure 2). From Figure 2(a) and (b), it can be seen that the genotoxicity of chlorinated wastewater D and E did increase obviously with an increasing NH_3−N concentration. When NH_3−N was low (<10∼20 mg/L), the genotoxicity after disinfection was lower than that before disinfection, and when NH_3−N was high (>10∼20 mg/L), the genotoxicity after disinfection was higher than that before disinfection.

TABLE 1. Characteristics of Wastewater Samples Used in This Study

samples	pH	COD (mg/L)	NH_3−N (mg/L)	DOC (mg/L)	UV_{254} (m^{-1})	genotoxicity[a] (μg 4-NQO/L-sample)
A(AAO)	8.3	10.2	1.1	5.6	16.4	13.8 ± 0.7
B(AS)	8.1	12.0	23.3	10.6	15.9	4.5 ± 0.6
C(AS)	7.8	11.5	14.6	5.7	14.2	10.1 ± 0.4
D(AS)	7.9	6.1	4.5	7.4	13.3	20.9 ± 2.9
E(MB)	8.3	6.7	2.7	5.7	12.9	12.5 ± 1.4
F(AS)	8.2	24.4	3.8	16.8	15.1	56.5 ± 3.7
G(MB)	7.8	10.4	0.9	7.0	13.8	5.9 ± 0.5

[a] Mean ± Standard deviation (based on triplicate analyses). AS, activated sludge process; AAO, anaerobic-anoxic-oxic process; MB, membrane bioreactor.

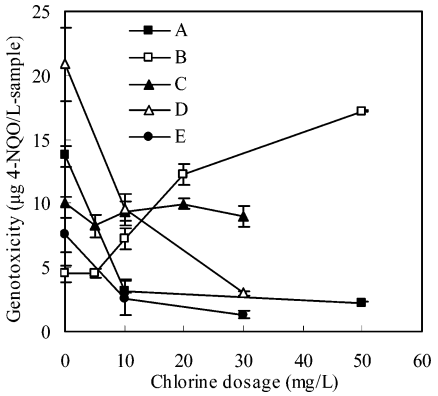

FIGURE 1. Change in genotoxicity of wastewater samples after chlorination with differing chlorine dosages. Error bars represent the standard deviation based on triplicate analyses. Characteristics of samples A∼E are shown in Table 1.

TABLE 2. DOC and UV_{254} of the Fractions of Samples F and G[a]

	sample F		sample G	
	DOC (mg/L)	UV_{254} (m^{-1})	DOC (mg/L)	UV_{254} (m^{-1})
HIS	5.5	7.9	4.1	8.8
HOA	5.1	5.3	1.6	4.6
HON	2.7	1.8	0.7	0.5
HOB	3.5	1.5	0.6	0.1

[a] HIS, hydrophilic substances; HOA, hydrophobic acids; HON, hydrophobic neutrals; HOB, hydrophobic bases.

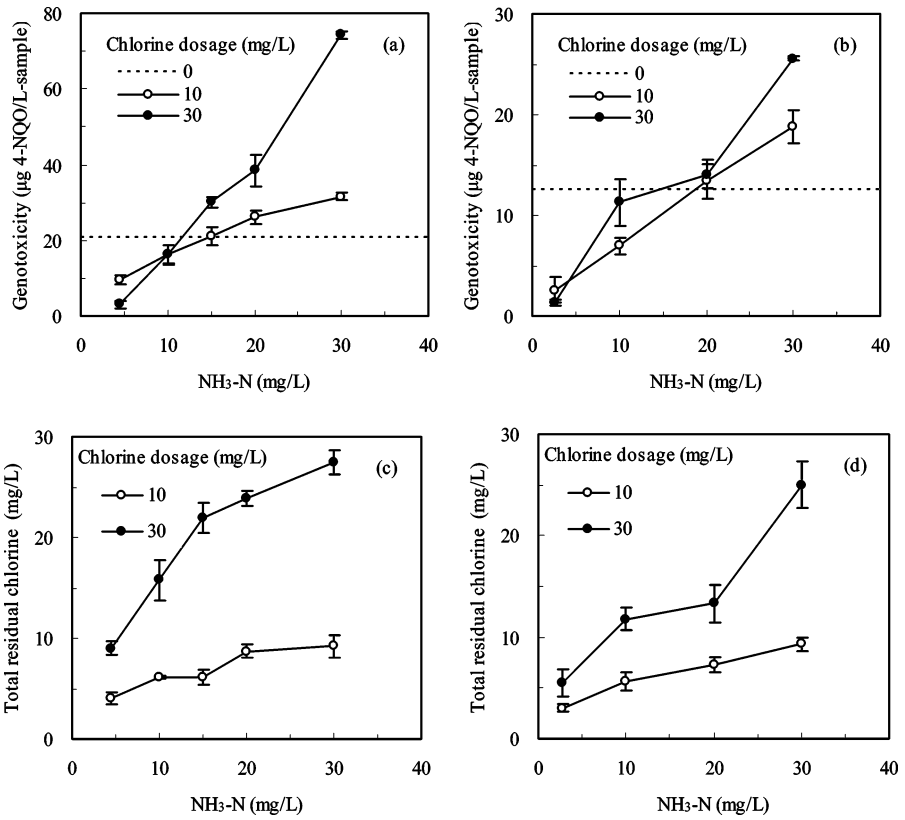

FIGURE 2. Changes in the genotoxicity and total residual chlorine of samples D and E with altered ammonia nitrogen concentrations after chlorination. (a) Genotoxicity of sample D, dashed line represents the genotoxicity of sample D before chlorination. (b) Genotoxicity of sample E, dashed line represents the genotoxicity of sample E before chlorination. Different scales are used on the vertical axes of (a) and (b) panels. (c) Total residual chlorine of sample D. (d) Total residual chlorine of sample E. Error bars represent the standard deviation based on triplicate analyses.

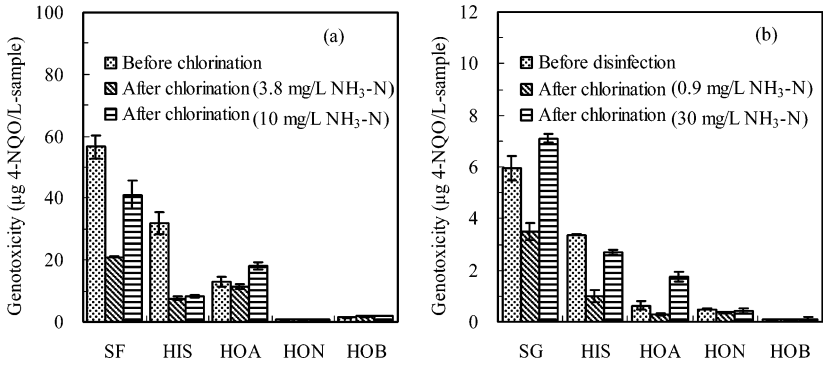

FIGURE 3. Changes in genotoxicity after chlorination of sample F, sample G and their fractions following the addition of differing amounts of ammonia nitrogen. (a) Genotoxicity of sample F and its fractions. (b) Genotoxicity of sample G and its fractions. SF, sample F without fractionation; SG, sample G without fractionation; HIS, hydrophilic substances; HOA, hydrophobic acids; HON, hydrophobic neutrals; HOB, hydrophobic bases. Error bars represent the standard deviation based on triplicate analyses.

During chlorination, ammonia nitrogen quickly reacted with free chlorine and changed it to combined chlorines (chloramines). Because the reactivity of combined chlorines is much weaker than that of free chlorine, combined chlorines were more slowly consumed during the reaction with DOM in wastewater. This is the reason why the total residual chlorine after 30 min chlorination increased with an increasing NH_3—N concentration as shown in Figure 2(c) and (d). Because of the weak reactivity of combined chlorines, ammonia nitrogen was reported to decrease the formation of typical DBPs, such as THMs and HAAs during chlorination of drinking water and wastewater (*16*, *17*). However, the results in this study suggest that disinfection with free chlorine under a low NH_3—N concentration could reduce the genotoxicity of wastewater, while disinfection of the wastewater with free chlorine under a high NH_3—N concentration might increase the genotoxicity.

It should be noted that a recently discovered DBP, iodoacetic acid, found in U.S. drinking water treated with chloramines turned out to be the most toxic DBP ever found (*18*). Choi (*19*) pointed out that chloramines could produce N-nitrosodimethylamine as a DBP. Zheng (*20*) reported that chlorination of publicly owned treatment works secondary effluent containing residual ammonia could lead to chloramination of organic compounds and the resulting production of cyanogen chloride and free cyanide. All of these

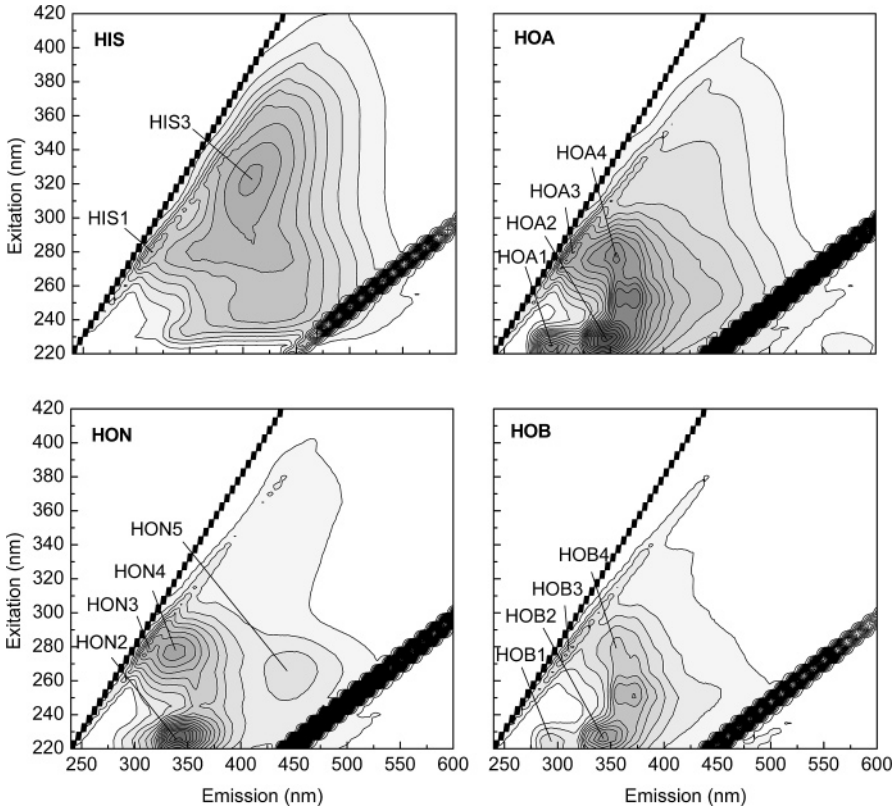

FIGURE 4. Fluorescence spectroscopy of fractions of sample F before chlorination. HIS, hydrophilic substances; HOA, hydrophobic acids; HON, hydrophobic neutrals; HOB, hydrophobic bases.

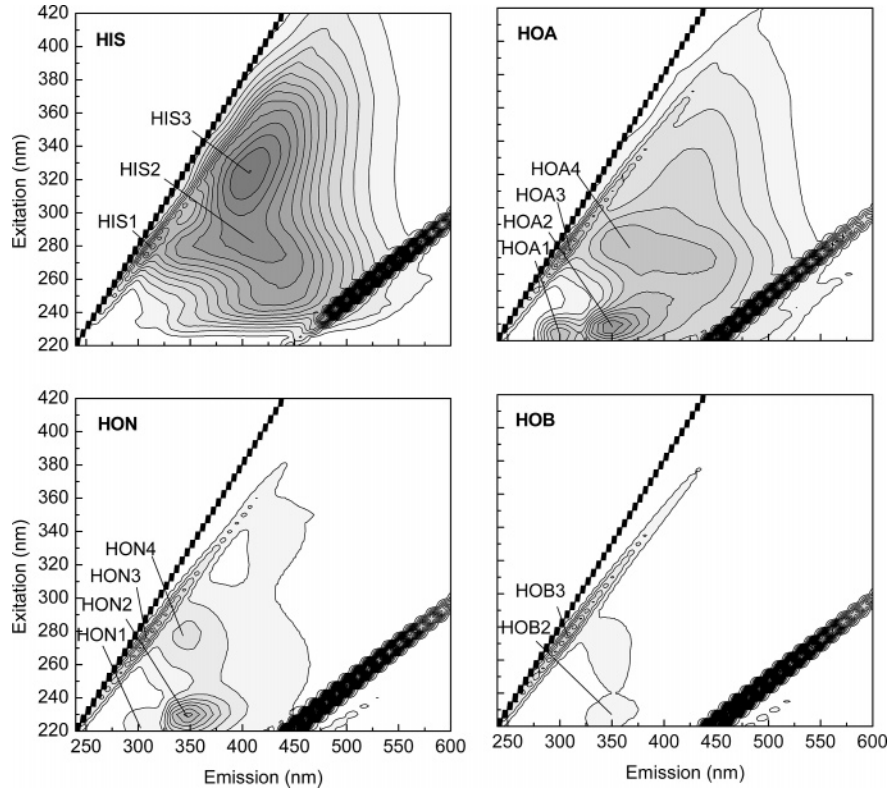

FIGURE 5. Fluorescence spectroscopy of fractions of sample G before chlorination. HIS, hydrophilic substances; HOA, hydrophobic acids; HON, hydrophobic neutrals; HOB, hydrophobic bases.

findings warned us that chlorination with a high NH_3—N concentration or chloramination of wastewater might be potentially harmful to the ecological system.

Effect of Chlorination on the Change in Genotoxicity of Different DOM Fractions. In order to discover the main precursors in causing the changes in wastewater genotoxicity,

TABLE 3. EEM Peaks for the Fractions of Samples F and G

	sample F		sample G		
peak	Ex_{max}/Em_{max} (nm/nm)	Intensity at Ex_{max}/Em_{max} (AU)	Ex_{max}/Em_{max} (nm/nm)	Intensity at Ex_{max}/Em_{max} (AU)	substance
HIS1	280/314	708.5	275/308	969.2	soluble microbial byproduct-like
HIS2			285/412	1173.0	humic/fulvic acid-like
HIS3	320/405	922.4	325/408	1400.0	humic/fulvic acid-like
HOA1	225/295	1312.8	225/301	690.8	tyrosine-like, aromatic protein
HOA2	230/347	1870.1	230/349	1101.0	tryptophan-like, aromatic protein
HOA3	280/315	1028.8	275/307	859.3	soluble microbial byproduct-like
HOA4	275/355	1225.3	280/364	694.5	soluble microbial byproduct-like
HON1			225/306	186.8	tyrosine-like, aromatic protein
HON2	225/341	1635.0	230/348	837.7	tryptophan-like, aromatic protein
HON3	280/314	893.4	275/308	671.1	soluble microbial byproduct-like
HON4	275/338	962.5	280/348	337.7	soluble microbial byproduct-like
HON5	265/446	376.6			humic/fulvic acid-like
HOB1	225/294	389.0			tyrosine-like, aromatic protein
HOB2	225/342	1058.0	225/343	162.1	tryptophan-like, aromatic protein
HOB3	275/308	528.5	275/308	609.0	soluble microbial byproduct-like

samples F and G were fractionated into four fractions. For both sample F and sample G, HIS and HOA were dominant in the DOC and UV_{254} value (Table 2). DOC is typically used as an aggregate measure for the content of organic matters and UV_{254} mainly reflects the content of unsaturated aromatic organics which are able to react with chlorine and produce DBPs (*21*). Therefore, HIS and HOA were considered to be the two main parts of DOM which might result in the change in genotoxicity.

With the aim of studying the effect of ammonia nitrogen on changes in genotoxicity during chlorination, NH_4Cl was added to sample F and each of its fractions to get an NH_3—N concentration of 3.8 mg/L (the same as that in sample F) and 10 mg/L, and NH_4Cl was added to sample G and each of its fractions to get an NH_3—N concentration of 0.9 mg/L (the same as that in sample G) and 30 mg/L. After the samples were chlorinated with 10 mg/L of chlorine for 30 min, the genotoxicity was measured as shown in Figure 3.

From Figure 3(a) and (b), it can be clearly seen that the change in genotoxicity for samples F and G was consistent with the previous result in this paper. For the four fractions from sample F and sample G, the genotoxicity of HIS and HOA was higher than that of HON and HOB before chlorination, and during chlorination, the change in genotoxicity of HIS and HOA was greater than that of HON and HOB, which proves the conclusion above that HIS and HOA are the two main fractions causing the change in genotoxicity. After chlorination with a low NH_3—N concentration, the genotoxicity of both HIS and HOA decreased noticeably, while when NH_3—N increased to a relatively high concentration, the genotoxicity of HIS still decreased, but the genotoxicity of HOA increased noticeably. This indicated that HIS played a key role in the decrease of genotoxicity during chlorination with a low NH_3—N concentration and HOA played a key role in the increase of genotoxicity during chlorination with a high NH_3—N concentration.

Fluorescence Spectroscopy of the Different DOM Fractions. In order to find out why the genotoxicity of different fractions changed so nonuniformly, EEM was used to characterize the fractions of samples F and G. The contour maps of the results are shown in Figure 4 and Figure 5. It can be seen that the there were different peaks for different fractions. The position of Ex_{max}/Em_{max} and the intensity at Ex_{max}/Em_{max} for these peaks is shown in Table 3. The substances which the peaks represent are also listed in Table 3 according to the literature (*22*).

From Table 3, it was found that HIS contained more humic/fulvic acid than other fractions, and HOA contained more aromatic protein than other fractions. Since some amino acids were reported to produce much more cyanogen halides when NH_3—N concentrations are higher (*23*), it is possible for some of the free or combined amino acids to produce more genotoxic byproducts when NH_3—N concentrations are higher and thus result in an increase in genotoxicity.

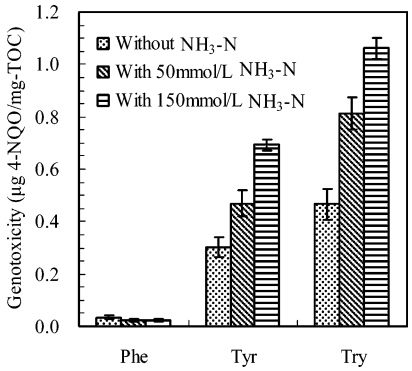

FIGURE 6. Effect of chlorination on the genotoxicity of three aromatic amino acids in the presence of different amounts of NH_3—N. Phe, Phenylalanine; Tyr, Tyrosine; Try, Tryptophan. Error bars represent the standard deviation based on triplicate analyses.

Genotoxicity of Twenty Amino Acids after Chlorination With and Without NH_3—N. Twenty amino acids: glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, serine, proline, threonine, cysteine, asparagine, glutamine, lysine, histidine, arginine, aspartate, and glutamate were dissolved in MilliQ water to a concentration of 0.1 mmol/L and NH_4Cl was added to get NH_3—N concentrations of 0, 50, and 150 mmol/L. Then all the solutions were chlorinated with 10 mmol/L chlorine for 30 min, and the genotoxicity of the chlorinated amino acids was measured. Only three aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Try) displayed an obvious genotoxicity after chlorination (see Figure 6). It could be seen that when NH_3—N concentration was higher, the genotoxicity of Phe did not change much, while the genotoxicity of Tyr and Try increased noticeably. These results validated the supposition that after chlorination, more genotoxic chlorinated amino acids could be formed when NH_3—N concentration was higher. Furthermore, because some PBTA (2-phenylbenzotriazole) compounds, another kind of chlorinated amines, were also found to be highly genotoxic (*24*), further efforts on genotoxicity evaluation and

chemical identification of chlorinated amines should be performed.

Acknowledgments

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