Combined genotoxicity of chlorinated products from tyrosine and benzophenone-4

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HIGHLIGHTS

- Genotoxicity of chlorinated mixture (GCM) is not predicted by that of single (GCI).
- pH is an important factor affecting difference between GCM and GCI ($G_{\Delta} = \text{GCM} - \text{GCI}$).
- $G_{\Delta} > 0$ occurred at pH 5.0–6.1 and $G_{\Delta} < 0$ occurred at pH 6.3–8.0.
- G_{Δ} is determined by N-DBPs decrease in mixture and combined effects between DBPs.
- TON ratio can be used to estimate the G_{Δ} value.

GRAPHICAL ABSTRACT

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ABSTRACT

The toxicity of disinfection by-products (DBPs) from a single precursor was studied intensively. Here we examined the genotoxicity when two precursors (tyrosine (Tyr) and benzophenone-4 (BP-4)) were chlorinated together and separately. We sought to examine whether the genotoxicity of the mixture (GCM) could be estimated from the sum of the genotoxicities of the individual precursors (GCI), which were chlorinated separately. We determined the genotoxicity using the SOS/umu test. The results revealed that GCM was not identical to GCI. The difference in genotoxicity between GCM and GCI (G_{Δ}) was observed to decrease with increasing pH. GCM was higher than GCI ($G_{\Delta} > 0$) at pH 5.0–6.1, and lower than GCI ($G_{\Delta} < 0$) at pH 6.3–8.0. We found that nitrogen-containing DBPs played a dominant role in determining GCM and GCI. We propose that the total organic nitrogen (TON) ratio, $\text{TON}_{(\text{chlorinated mixture})} / \text{TON}_{(\text{the sum of chlorinated individuals})}$, is useful to estimate G_{Δ} .

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1. Introduction

Chlorine is one of the most commonly used disinfectants in drinking water. However, disinfection by-products (DBPs, Acronyms used in this paper is listed in Box 1) with genotoxic, mutagenic and carcinogenic activities are produced during chlorination. In fact, chlorine reacts with natural organic matter (e.g., humic/fulvic acids, proteins and amino acids) [1–3], anthropogenic chemicals (e.g., pesticides and personal care products) [4], and salts (e.g., bromide and iodide) [5], and produces a wide variety of DBPs [6]. Although most DBPs' concentrations are very low (~ 1 – $100 \mu\text{g/L}$) [7], they may pose a human health hazard as a result of persistent exposure and/or their synergistic effects [8–10]. Many previous studies have focused on the toxicity of a single DBP [2], a single chlorinated compound [11–13] or chlorinated water samples [14–16]. However, when multiple precursors are chlorinated, little is known about the factors that cause the toxicity of the mixture and whether the mixture's toxicity can be estimated from the sum of the toxicities of the individual chlorinated precursors. Accordingly, we need to determine how and why the toxicity varies during chlorination of multiple precursors.

The composition of source water is complicated. Thus it is difficult to study the underlying mechanism of combined genotoxicity of DBPs from multiple precursors. To simplify the study, we selected two representative precursors, tyrosine (Tyr, a natural organic matter) and benzophenone-4 (BP-4, an anthropogenic chemical). Tyr is a naturally occurring amino acid and is present in many peptides, proteins, and algae [17,18]. The largest influent concentration of the hydrolysable Tyr is $27.4 \mu\text{g/L}$ [19]. Its DBPs were identified and quantified in previous studies [20–23]. BP-4 is one of the most widely used UV sunscreens and is used in a variety of personal care products (e.g., sunscreens, lipsticks, lotions, shampoos and cosmetics) [24–26]. BP-4 has been detected in wastewater, river water, and sea water, at concentrations ranging from ng/L to high $\mu\text{g/L}$ levels [27,28]. However, traditional wastewater treatment plants did not efficiently eliminate BP-4. Moreover, BP-4 could be present in reclaimed water. Consequently, it is important to determine BP-4 byproducts that are formed during chlorination [13]. Both Tyr and BP-4 are common precursors in reclaimed source waters. Furthermore, Tyr is a precursor of nitrogen disinfection by-products (N-DBPs) [20], and BP-4 reacts with disinfectants only to form carbonaceous by-products (C-DBPs) [13]. In vivo genotoxicity and cytotoxicity assays suggest that the genotoxic and cytotoxic activities of N-DBPs are higher than those of C-DBPs [29–31]. Accordingly, curiosity in the study of N-DBPs has increased rapidly.

Accordingly, we hypothesized that after chlorination, the toxicity of a Tyr and BP-4 mixture could not be equal to the sum of individual toxicities that result from chlorinating them separately. In this study, we used genotoxicity to test this hypothesis and then explore: (i) what is the main experimental condition affecting the difference between the genotoxicity of the chlorinated mixture (GCM) and the sum of genotoxicities of the individual chlorinated compounds (GCI), (ii) what is the underlying mechanism of the genotoxicity difference between GCM and GCI (G_{Δ} , $G_{\Delta} = \text{GCM} - \text{GCI}$), (iii) develop a methodology to estimate G_{Δ} .

2. Materials and methods

2.1. Chemicals and solution preparation

Standards for the analysis of the following DBPs were purchased from AccuStandard (USA): (1) chloroform (CF) and (2) halo-generated volatile organic chemical as a mixture of DBPs including dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), chloral hydrate (CH), chloropicrin (CP), 1,1-dichloropropanone (DCP) and 1,1,1-trichloro-2-propanone (TCP). Two species of haloacetic acids (HAAs, including dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA)) were purchased from Bei Na Chuang Lian Ltd. (China). Dichloroacetamide (DcAcAm) and trichloroacetamide (TcAcAm) were purchased from Alfa Aesar (Germany) and J&K (China), respectively. Ethyl acetate and methyl *tert*-butyl ether (MTBE) used to extract DBPs were obtained from Fisher (USA). Tyr was obtained from Sigma-Aldrich (USA). BP-4 and phloretic acid (PA) were obtained from J&K (China). The chemical structures of Tyr, BP-4 and PA were listed in Fig. 1. The sodium hypochlorite aqueous solution was obtained from Sinopharm Chemical Reagent Co., Ltd (China). The concentration of the chlorine stock solution was determined using the *N,N*-diethyl-*p*-phenylenediamine ferrous titration method. All chemicals were of analytical grade and used without further purification. All reagent solutions were prepared with ultrapure water (resistivity $18.2 \text{ M}\Omega \text{ cm}$, Millipore, US). Additionally, all bottles were rinsed three times with ultrapure water and then dried in a muffle furnace at 500°C for 4 h.

2.2. Chlorination experiments

Chlorination experiments were performed under headspace-free conditions in glass screw-cap vials that were capped with Teflon-faced septa and kept in the dark. Hydrochloric acid and sodium hydroxide were used to adjust the pH ($\text{pH} = 5.0$ – 8.0), 50 mM

Box 1: Acronyms used.

DBPs	Disinfection by-products	TCP	1,1,1-trichloro-2-propanone
Tyr	tyrosine	HAAs	haloacetic acids
BP-4	Benzophenone-4	DCAA	dichloroacetic acid
GCM	genotoxicity of chlorinated mixture	TCAA	trichloroacetic acid
GCI	the sum of genotoxicity of chlorinated individual precursor	DcAcAm	Dichloroacetamide
G_{Δ}	the difference between genotoxicity of chlorinated mixture and the sum of genotoxicity from chlorinated individual precursor	TcAcAm	trichloroacetamide
TON	total organic nitrogen	MTBE	methyl <i>tert</i> -butyl ether
N-DBPs	nitrogen-containing DBPs	Cl_2	chlorine
C-DBPs	carbonaceous by-products	PA	phloretic acid
CF	chloroform	GC/MS	gas chromatography/mass spectrometry
DCAN	dichloroacetonitrile	UPLC/DAD	ultra-performance liquid chromatography/diode array detector
TCAN	dichloroacetonitrile	Q-TOF-MS	Quadrupole-Time of Flight Mass Spectrometer
CH	chloral hydrate	TKN	Total Kjeldahl nitrogen
CP	chloropicrin	$\text{TEQ}_{4\text{-NQO}}$	Toxicity Equivalent Quotient
DCP	1,1-dichloropropanone	HOCl	hypochlorous acid
SD	Supplementary data

Fig. 1. Chemical structures of the tested compounds.

of phosphate buffer solution was applied to maintain the desired pH levels for pH values of 6.0 to 8.0, and 100 mM phosphate buffer solution was used to adjust the desired pH level at pH = 5.0. To easily determine the concentrations and genotoxicity of chlorination byproducts from Tyr and BP-4, the initial concentrations of precursors were designed as 0.1 mM in the experiments. The experiments were conducted in five phases. For phase 1, to ensure excess chlorine during chlorination, 3 mM solution of free chlorine (Cl₂) was added to 200 mL solutions of Tyr (0.1 mM), BP-4 (0.1 mM) and a mixture (0.1 mM of Tyr and 0.1 mM of BP-4) (Fig. S1(a), Supplementary data (SD)). (These results allowed us to determine whether the GCM varies relative to GCI and the primary environmental factors affecting G_{Δ} .) For phase 2, there was a measurable chlorine residual after 72 h of chlorination when the initial molar concentration of chlorine ([Cl₂]₀) was 15 times as much as that of precursors ([Tyr]₀, [BP-4]₀ and [mixture]₀) (Fig. S1(b), SD). To confirm at which time the G_{Δ} is the largest, varying reaction time experiments were conducted. Reaction times were 10 min, 1 h, 6 h, 24 h and 72 h. For total mixture concentrations of 0.1 mM, these molar ratios of [Tyr]₀: [BP-4]₀ were chlorinated using 1.5 mM of free chlorine at room temperature (25 ± 0.2 °C): 0:1, 2:8, 5:5, 8:2 and 1:0. (These results allowed us to determine which molar ratio yields the largest G_{Δ} > 0.) For phase 3, Tyr, BP-4 and a mixture of the two ([Tyr]₀: [BP-4]₀ = 8:2, which corresponded to a mixture investigated in phase 2) were chlorinated for several narrow pH intervals at room temperature (25 ± 0.2 °C). (These results allowed us to determine the impact of pH on G_{Δ} .) For phase 4, Tyr with and without ammonium chloride (NH₄Cl), and PA-BP-4 mixtures (0.1 mM, [PA]₀: [BP-4]₀ = 2:8, 5:5, 8:2 and 1:0) were chlorinated at pH = 6.0 at room temperature (25 ± 0.2 °C), respectively. (These results allowed us to determine the primary DBPs affecting GCM and GCI.) For phase 5, 0.08 mM of Tyr and 0.02 mM of BP-4 were chlorinated separately. The post-chlorination samples were mixed (hereafter we referred to them as the post-chlorination mixture), followed by genotoxicity determination. (These results allowed us to determine the combined effects.) To completely quench the chlorine residual, ascorbic acid was used, and the dosage was double the initial molar concentration of chlorine. All experiments were conducted in triplicate.

2.3. Identification of transformation products

The reaction byproducts of Tyr were analyzed using gas chromatography/mass spectrometry (GC/MS, Agilent, USA) with an electron impact source (Agilent, USA) [32]. The transformation products of BP-4 were analyzed by a Waters ultra-performance liquid chromatography/diode array detector (UPLC/DAD) system equipped with a Quadrupole-Time of Flight Mass Spectrometer (Q-TOF-MS) (USA) [13]. The experimental methods are presented in Text S1.

2.4. DBP analysis

CF, CH, CP, DCP, TCP, DCAN and TCAN were extracted using MTBE and analyzed by USEPA's method 551.1 [33]. Two species of HAAs, including DCAA and TCAA, were methylated using acidic methanol, followed by extraction using MTBE, and measured using USEPA method 552.3 [34]. DcAcAm and TcAcAm were analyzed by GC/MS [23]. More analysis details are described in Text S2.

2.5. Determination of total organic nitrogen (TON)

Concentrations of TON were estimated by subtracting the ammonia nitrogen (NH₃-N) concentration from the total Kjeldahl nitrogen (TKN) concentration. The TKN concentrations were determined by the modified nitrogen method [35] using a Digestion Unit K-435 and AutoKjeldahl Unit K-370 (China). Concentrations of NH₃-N were measured by the Nessler's reagent colorimetric method [36] using a U-3010 UV-vis spectrophotometer (Hitachi Co, Japan) equipped with 10 mm quartz cuvettes. More experimental details were listed in Text S3 of SD.

2.6. Genotoxicity test

To evaluate the genotoxic responses to Tyr, BP-4 and their mixture with and without chlorination, a modified SOS/umu test [37] using *Salmonella typhimurium* TA1535/pSK1002 without S9 activation was used. The SOS/umu assay has been shown to be useful as a biological endpoint for genotoxicity monitoring of DBPs containing unknown genotoxic substances [12,38,39]. Prior to genotoxicity measurement, the samples were concentrated using two pre-conditioned Oasis HLB solid extraction cartridges (6 mL 500 mg, Waters, USA). The extracts were then dissolved into 200 μL of dimethyl sulfoxide. The genotoxic activities of samples were qualitatively and quantitatively expressed by the induction factor and the equivalent 4-nitro-1-oxide (4-NQO) concentration (Toxicity Equivalent Quotient, TEQ_{4-NQO}), respectively. In genotoxicity tests, all samples were evaluated in triplicate. The experimental procedure is described in Text S4.

2.7. Statistical analysis

The Pearson correlation coefficient was determined using SPSS 19.0 (IBM) and the relationships between GCM and the concentrations of DBPs were determined. The independent sample T-test was carried out to determine if GCM and the genotoxicity of the post chlorination mixture were significantly different from GCI. The T-test was also used to determine if there were any significant genotoxicity differences for chlorinated Tyr with NH₄Cl compared to chlorinated Tyr alone, as well as for the chlorinated Tyr and BP-4 mixtures ([Tyr]₀: [BP-4]₀ = 2:8, 5:5, 8:2 and 1:0) compared to chlori-

Fig. 2. The variation of GCM and GCI at pH 5.0–8.0 after 24-h chlorination ([Tyr]₀: [BP-4]₀ = 8:2, T = 25 °C, [Cl₂]₀ = 1.5 mM). Inset shows G_Δ at pH 5.0–8.0. Asterisks indicate that GCM is significantly different from GCI ($p < 0.05$). Error bars represent the standard deviation of replicate measurements ($n = 3$).

nated PA and BP-4 mixtures ([PA]₀: [BP-4]₀ = 2:8, 5:5, 8:2 and 1:0). A non-linear regression analysis was used to determine the relationship between G_Δ and TON ratio. Statistical significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Occurrence of G_Δ

We found that both Tyr and BP-4 alone or as a mixture did not exhibit genotoxicity (Fig. S2). After chlorination, we observed genotoxic responses (Fig. S3), due to the formation of genotoxic byproducts [13,40]. Furthermore, we found that GCM was not equal to GCI. G_Δ varied with reaction pH, time, temperature and chlorine dosage. It was observed that GCM was significantly higher than GCI at pH = 6.0. Hence, it was great significant to study the effect of pH on G_Δ.

3.2. The influence of pH on G_Δ

To further investigate the influence of pH, we conducted experiments with different molar ratios of Tyr and BP-4 and determined the G_Δ values (Text S5). We found that a Tyr-to-BP-4 molar ratio of 8:2 (Fig. S4) had the largest G_Δ; consequently, we conducted additional research on this molar ratio.

For pH values from 5.0 to 8.0, the maximum G_Δ occurred at 24 h. (Fig. S5, SD). Accordingly, we examined the G_Δ in a narrow pH interval after 24 h of chlorination. A significant decrease in G_Δ was observed with increasing pH from 5.0 to 8.0 (Fig. 2, inset). G_Δ > 0 occurred at pH values 5.0–6.1, while G_Δ was less than 0 at pH 6.3–8.0. Additionally, GCM and GCI decreased with increasing pH (Fig. 2). This can be explained by the impact of pH on the species distribution of hypochlorous acid (HOCl) and hypochlorite (OCl⁻) and the reactivity of chlorine. In acidic conditions, HOCl is the dominant oxidant species ($pK_a = 7.54$), and HOCl will open the ring of a substituted benzene. Ring opening increases the formation of DBPs that are thought to be more genotoxic [2].

3.3. The underlying mechanism of G_Δ

Based on GC/MS and UPLC/MS methods, the chlorinated products of Tyr and BP-4 alone and their mixture ([Tyr]₀: [BP-4]₀ = 8:2)

were measured at pH 6.0 (Fig. S6, SD). The species of the byproducts that were formed by Tyr and BP-4 when they were chlorinated individually were the same as those in their mixture ([Tyr]₀: [BP-4]₀ = 8:2). This revealed that the reaction pathway of Tyr and BP-4 in mixture was basically identical to that for the individual precursors. To explore the underlying mechanism of G_Δ, it was crucial to determine the dominant DBPs affecting GCM and GCI and determine their combined effects.

3.3.1. Dominant DBPs affecting the GCM and GCI

To determine the primary DBPs that contribute to the genotoxicity, the possible C- and N-DBPs were measured (Fig. S7) [20]. We found that the four C-DBPs, including CF, CH, DCAA and TCAA, were not correlated ($p > 0.05$) with GCM and GCI. This suggested C-DBPs make a small contribution to GCM and GCI. However, both DCAN and TcAcAm were correlated with GCM Fig. S8 (a), (DCAN ($R^2 = 0.411$, $p < 0.05$), TcAcAm ($R^2 = 0.701$, $p < 0.01$)) as well as with GCI Fig. S8 (b), (DCAN ($R^2 = 0.621$, $p < 0.01$), TcAcAm ($R^2 = 0.630$, $p < 0.01$)). The DCAN and TcAcAm genotoxicity (TEQ_{4-NQO} value: 1.7×10^{-4} µg/L and 2.5×10^{-4} µg/L, respectively) accounted for a tiny fraction of N-DBP genotoxicity. To investigate the role of unknown N-DBPs in GCM and GCI, we compared the genotoxicity of Tyr with NH₄⁺ and that without NH₄⁺ (Fig. 3(a)), as well as the genotoxicity of Tyr and that of PA the same structure with Tyr no amine group (Fig. 3(b) and (c)) (Text S6). Results suggested that N-DBPs dominated GCM and GCI. Because G_Δ reflected the genotoxicity difference between GCM and GCI, G_Δ was related to the N-DBP production in the mixture as compared to the sum of the individual chlorinated precursors.

3.3.2. Combined effects between DBPs of Tyr and BP-4

The chlorination products of Tyr and BP-4 may interact to produce a synergistic or antagonistic impact on G_Δ. To investigate this, we determined the genotoxicity of the post-chlorination mixture and compared it with the GCI. We found that the post-chlorination mixture had a significantly higher genotoxicity for pH values from 5.0 to 7.0 and a lower genotoxicity at pH 8.0 than the 24-h GCI (Fig. 4). This suggested that the combined effects occur between the chlorinated products of Tyr and BP-4, and they played an important determining role in the resulting G_Δ. Accordingly, G_Δ depends on not only N-DBP production between the mixture and individual precursors but also the combined effects between DBPs from Tyr and BP-4.

3.3.3. The role of N-DBPs and combined effects in G_Δ

To evaluate the importance of N-DBP production and the impacts on G_Δ, TON concentrations were measured as a surrogate to estimate the total concentration of N-DBPs. Table 1 showed that the N-DBP production in the mixture was lower than the sum of production by the individual precursors. Nevertheless, G_Δ was greater than 0 for pH values from 5.0 to 6.1 (Fig. 2), and the above results showed a synergistic effect in the post-chlorination mixture at pH 5.0–7.0 (Fig. 4). Based on comparison of the TEQ_{4-NQO} values from the chlorinated mixture (11.9 µg/L at pH 5.0 and 6.6 µg/L at pH 6.0) and the post-chlorination mixture (9.5 µg/L at pH 5.0 and 6.3 µg/L at pH 6.0), we concluded that synergistic effects were the main cause for G_Δ > 0. However, G_Δ > 0 could not be fully explained by synergistic effects. Differences in the N-DBP composition might also contributed to the occurrence of G_Δ > 0.

In contrast, G_Δ was observed to be less than 0 for pH values from 6.3 to 8.0 (Fig. 2). We found that this was related to a decrease in N-DBPs that were produced from the mixture as compared to the sum of those from the individual chlorinated precursors (Table 1). As pH increased from 5.0 to 8.0, N-DBPs decreased significantly in the mixture (Table 1), and the synergistic effect decreased, and then an antagonistic effect took place (Fig. 4). The decrease of N-DBPs in

Table 1

TON concentrations in chlorinated Tyr and BP-4 mixture and in chlorinated individuals and the TON ratio.

pH values	[Tyr] ₀ : [BP-4] ₀	M _{TON} (mg/L)	I _{TON} (mg/L)	TON ratio
pH = 5.0	8:2	0.856 ± 0.01	0.944 ± 0.02	0.911 ± 0.02
pH = 6.0	2:8	0.062 ± 0.01	0.106 ± 0.01	0.572 ± 0.01
	5:5	0.131 ± 0.02	0.265 ± 0.01	0.491 ± 0.02
	8:2	0.274 ± 0.02	0.424 ± 0.01	0.637 ± 0.03
pH = 7.0	8:2	0.135 ± 0.01	0.408 ± 0.01	0.331 ± 0.02
pH = 8.0	8:2	0.050 ± 0.01	0.232 ± 0.01	0.216 ± 0.04

Note: M_{TON} represents the TON concentrations of chlorinated Tyr and BP-4 mixture and I_{TON} represents the sum of the TON concentration of chlorinated single precursor.

$I_{TON(2:8)} = 0.2 M_{TON(1:0)} + 0.8 M_{TON(0:1)}$.

$I_{TON(5:5)} = 0.5 M_{TON(1:0)} + 0.5 M_{TON(0:1)}$.

$I_{TON(8:2)} = 0.8 M_{TON(1:0)} + 0.2 M_{TON(0:1)}$.

TON ratio = $M_{TON(x:y)} / I_{TON(x:y)}$ (x,y) = (2:8), (5:5) and (8:2) $M_{TON(0:1)} = 0$.

Error bars represent the standard deviation of replicate measurements (n = 3).

the mixture rather than a synergistic effect played a leading role in G_{Δ} being less than 0 at high pH. Accordingly, the value of G_{Δ} was determined by the combined impacts of synergistic or antagonist effects of the DBPs and the decrease of N-DBPs in the mixture.

3.4. Estimation of G_{Δ} by TON ratio

In this study, the reaction pathway of N-DBPs was consistent in the chlorination of Tyr alone and in mixture. The TON ratio, defined as $TON_{(chlorinated\ mixture)} / TON_{(the\ sum\ of\ chlorinated\ individuals)}$,

was adopted to estimate G_{Δ} (Table 1). A non-linear regression analysis suggested that there was a significant correlation between G_{Δ} and TON ratio ($R^2 = 0.997$, $p < 0.01$) (Fig. 5). The regression equation ($y = 18.5x^2 - 9.9x + 0.9$) could be used to estimate G_{Δ} .

Remarkably, all TON ratios were lower than 1, indicating the addition of BP-4 decreases the production of N-DBPs from Tyr. On the basis of previous literature, chlorination reactions with Tyr [41] and BP-4 [13] were described by following Eqs. (1) and (2)

$$-d[Tyr]/dt = k_{obs}[Tyr][Chlorine] \quad (1)$$

Fig. 3. (a) Effects of chlorination on the genotoxicity of mixtures containing different concentrations of Tyr and NH_4^+ (pH = 6.0, $T = 25^\circ C$, $[Cl_2]_0 = 1.5\text{ mM}$, $t = 24\text{ h}$). (b) Comparison of genotoxicity between chlorinated Tyr (0.1 mM, green bars) and chlorinated PA (0.1 mM, blue bars) after different chlorination times. (pH = 6.0, $T = 25^\circ C$, $[Cl_2]_0 = 1.5\text{ mM}$). (c) Comparison of genotoxicity between chlorinated Tyr/BP-4 mixture (0.1 mM, $[Tyr]_0:[BP-4]_0 = 2:8, 5:5$ and $8:2$; green bars) and chlorinated PA/BP-4 mixture (0.1 mM, $[PA]_0:[BP-4]_0 = 2:8, 5:5$ and $8:2$; blue bars) after different chlorination times (pH = 6.0, $T = 25^\circ C$, $[Cl_2]_0 = 1.5\text{ mM}$). Asterisks indicate that GCM ($[Tyr]_0:[BP-4]_0 = 2:8, 5:5, 8:2$ and $1:0$) is significantly different from the genotoxicity of chlorinated PA/BP-4 mixtures ($[PA]_0:[BP-4]_0 = 2:8, 5:5, 8:2$ and $1:0$) ($p < 0.05$). Error bars are the same as those in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Comparison of GCI and the genotoxicity of the post-chlorination mixture ($[\text{Tyr}]_0 = 0.08 \text{ mM}$, $[\text{BP-4}]_0 = 0.02 \text{ mM}$) at varying pH values ($T = 25^\circ\text{C}$, $[\text{Cl}_2]_0 = 1.5 \text{ mM}$, $t = 24 \text{ h}$). Asterisks indicate that the genotoxicity of the post-chlorination mixture is significantly different from GCI ($p < 0.05$). Error bars are the same as those in Fig. 1.

Fig. 5. The non-linear regression analysis of G_Δ at varying TON ratios ($T = 25^\circ\text{C}$, $[\text{Cl}_2]_0 = 1.5 \text{ mM}$, $t = 24 \text{ h}$). TON ratio was defined as $\text{TON}_{(\text{chlorinated mixture})} / \text{TON}_{(\text{the sum of chlorinated individual})}$. Error bars are the same as those in Fig. 1.

$$-d[\text{BP-4}]/dt = k'_{\text{obs}}[\text{BP-4}] \quad (2)$$

where k_{obs} , k'_{obs} represent the rate constants of Tyr and BP-4, respectively. k_{obs} (10^5) was observed to be approximately 3 to 4 orders of magnitude higher than k'_{obs} (10^1 – 10^2) [39,41]. Under the same chlorine dosage, compared with Tyr alone, Tyr in mixture could be reacted with more chlorine, thus accelerating the Tyr chlorination. Based on the reaction pathway of Tyr, N-DBPs are generated and then hydrolyzed to form C-DBPs [19]. During the contact time between 6 h and 72 h, N-DBPs were being degraded (Fig. S7). So Tyr in mixture produced less N-DBPs at 24 h as compared to Tyr alone. To verify the effect of chlorine dosage on the degradation of N-DBPs, the genotoxicity and TON concentrations of Tyr under various chlorine dosages ($[\text{Cl}_2]_0 : [\text{Tyr}]_0 = 7.5:1$, $15:1$ and $30:1$) were measured (Fig. 6). The decreases in genotoxicity and TON concentrations were statistically significant ($p < 0.01$) with increasing chlorine dosage. This result suggested that the TON ratio was lower than 1 because the formation of N-DBPs in mixture was lower than with Tyr alone. Moreover, the TON ratio decreased with increasing pH (Fig. 5). This may be associated with the pH dependence of k_{obs} and k'_{obs} . It has been proved that a rise in pH can lead to increases of k_{obs} and k'_{obs} [13,42], and the increase of k_{obs} is much larger than that of k'_{obs} . More chlorine reacted with Tyr in mixture at higher pH, thus decreasing the TON ratio. As a consequence, the pH dependence of the rate constant is responsible for the variation in the TON ratio.

Fig. 6. Genotoxicity and total organic nitrogen (TON) concentrations of chlorinated Tyr (0.1 mM) with different chlorine dosages ($\text{pH} = 6.0$, $T = 25^\circ\text{C}$, $t = 24 \text{ h}$). Asterisks indicate that the genotoxicity is significantly different for chlorinated Tyr with different chlorine dosages ($p < 0.05$). Error bars are the same as those in Fig. 1.

4. Conclusions

This study investigated the role of pH in controlling G_Δ . We confirmed that the genotoxicity of a chlorinated Tyr and BP-4 mixture could not be estimated from the sum of the genotoxicities of the individual chlorinated precursors. pH was an important influencing factor affecting G_Δ . G_Δ decreased with increasing pH values from 5.0 to 8.0. $G_\Delta > 0$ occurred at pH 5.0–6.1, and $G_\Delta < 0$ occurred at pH 6.3–8.0. Synergistic effects played a crucial role in the genotoxicity of the chlorinated mixture and induced a higher genotoxicity ($G_\Delta > 0$). A reduction of N-DBPs in the mixture caused G_Δ to be less than 0. Although the precursors in this study were single compounds (Tyr and BP-4), our study provided valuable insight regarding the genotoxicity of DBPs from other multiple precursors. It is expected that researchers will pay more attention to the genotoxicity difference between mixtures and individual precursors. In addition, the role of the N-DBPs and combined effects during the evaluation of genotoxicity differences is likely to be of particular concern.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2016.10.014>.

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