

## Toxicity measurements in aqueous solution during ozonation of mono-chlorophenols

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### Abstract

The Microtox toxicity and Oxygen Uptake Rate (OUR) inhibition tests were conducted to monitor the variation of toxicity during ozonation of 2-chlorophenol (2-CP), 3-chlorophenol (3-CP) and 4-chlorophenol (4-CP) under neutral conditions. The results revealed that the oxidized 2-CP solution exhibited new toxicity to pure bacteria and mixed microorganisms in the early stage of ozonation. The largest inhibition of OUR appeared at one mol of applied ozone dosage per mol of initial 2-CP, and the percentage of inhibition was 63.8%. In addition, ozonated 3-CP and 4-CP also significantly induced new aqueous toxicity, if these toxic intermediates were not further ozonated. Comparing the variation of toxicity and the hydroxylated/chlorinated intermediates formed, 3-chloro-catechol, 2-chloro-2, 4-hexadienedioic acid and the dimmer compounds may be related to the sources of toxicity during the ozonation of 2-CP.

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### 1. Introduction

Many aromatic compounds, including the chloro-, nitro-substituted aromatic compounds or substituted phenols have been reported to be quite bioresistant in nature and in treatment plants. One group of these compounds, the chlorophenols (CPs) is typically biorefractory or toxic in the aqueous environment due to their chloro substituent (Stefan et al., 1995). Related studies have examined the feasibility of using partial oxidation of chlorophenols by ozone or other oxidants (advanced oxidation processes, AOPs) to promote biodegradability for subsequent biological treatment and to reduce the toxicity of effluent (Trapido et al., 1997; Hu and Yu, 1994; Stockinger et al., 1995; Yu and Hu, 1994). In general, the byproducts formed in these oxidation processes are mainly low-molecular-weight (organic acids) or hydroxylated compounds, which are presumed to be more biodegradable or less toxic than their parent compounds (Miller et al., 1988; Lipczynska-Kochany

and Bolton, 1992). Therefore, Adams et al. (Adams et al., 1997) indicated that the ozonation of 4-CP and 2, 4-dichlorophenol (DCP) significantly enhanced their biodegradability by the unacclimated microbial seed obtained from a domestic wastewater treatment plant's activated sludge, and that the ultimate COD (chemical oxygen demand) removal increased about 50% for these two chlorophenols. Trapido et al. also reported that ozonation of CPs (2-CP, 4-CP, 4-chlororesorcinol, 6-chloro-m-cresol and 2, 4-dichlorophenols) decreases their toxicity according to the *Daphnia magna* 24 h toxicity test when ozone treatment leads to 90–95% removal of CPs.

On the other hand, the toxic intermediates of oxidative treatment prior to the detoxification of the chlorinated aromatic compounds have been reported (Hirvonen et al., 2000; Svenson and Hynning, 1997; Manilal et al., 1992). Hirvonen et al. reported that the hydroxylation of chlorophenols and formation of dimeric intermediates were involved in the oxidation of chlorophenols by ozone and UV/H<sub>2</sub>O<sub>2</sub> processes. Hydroxylated chlorophenols, chlorodihydroxybiphenyls and dichlorodihydroxybiphenyls were formed in both oxidation treatments of monochlorophenols (2-CP and 4-CP). These byproducts showed the characteristics of high molecular weight or the presence of

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chlorinated intermediates, which are of concern due to their toxicity. Svenson and Hynning indicated that photolytic degradation of 2, 4, 6-trichlorophenol was found to increase aqueous toxicity measured by the Microtox bioassay system, and this increasing toxicity was mainly generated from the 3, 5-dichlorocatechol formed. In light of the above developments, oxidation treatment of mono-chlorophenols by ozone is studied in this investigation, and attention is focused on the variation of toxicity. In this study, acute toxicity of pure CPs and ozonated solutions is assessed by the Microtox toxicity assay (Nirmalakhandan et al., 1994), and the inhibition of the activated sludge system is measured by Oxygen Uptake Rate (OUR).

## 2. Materials and methods

### 2.1. Process of ozonation

Fig. 1 shows a schematic diagram of the ozonation apparatus employed as a semi-batch type. Ozonation was performed in a lab-scale bubble column reactor with a magnetic stirrer. All the components in this system are made from acrylic glass, Teflon or stainless steel. The ozone generator was manufactured by JEU TU CO., Japan (Series No, SG-01A-PSA4). The ozone gas was introduced into a diffuser in the bottom of the reactor. The concentration of ozone gas was measured by the potassium iodide technique based on the standard methods (APHA, 1992). The mono-chlorophenols used in this study were 2-chlorophenol, 3-chlorophenol and 4-chlorophenol. They were obtained from Merck (reagent grade). The volume of the reaction solution was 1000 mL and Milli-Q water was used in all experiments. The pH was fixed at pH 7 in all ozonation

processes. A pH controller (SUNTEX, pH/ORP Controller PC-310), an adding pump (Cole-Parmer 7553-80) and 0.2N NaOH or H<sub>2</sub>SO<sub>4</sub> solution was utilized to control pH during ozonation. Table 1 lists other detailed operating conditions of ozonation.

The CPs concentration during ozonation was analyzed by HPLC (Bio-Rad 1350) equipped with a UV/VIS spectrophotometer (Bio-Rad 1706) employing a wavelength of 280 nm and a C18 reverse-phase column (Phenomenex). The mobile phase consisted of acetonitrile, water and acetic acid, regulated at a 580: 420: 5.7 by volume ratio. The concentration of chloride ion was measured by using a Dionex 2000i ion chromatograph (IC) with a Dionex AS4A anion separator column. A TOC analyzer (O-I-Analytical, model 700) was employed to measure the total organic carbon (TOC) of the ozonated solution. Chemical oxygen demand (COD) was analyzed according to the standard methods (APHA, 2350E, 1992).

### 2.2. Microtox assay

Acute toxicity of the pure compounds and solutions of their ozonated intermediates was measured by the Microtox toxicity technique, using *Photobacterium phosphoreum*. Analysis was conducted according to the standard Microtox test procedures using a Microtox analyzer (Model 500, Microbics Corp, Carlsbad, CA). In this paper, the EC<sub>50,15 min</sub> value (that is the effective concentration of the sample that causes 50% inactivation of the test bacteria in 15 min) was used to trace the change in toxicity during ozonation. The Microtox test was operated in duplicate with a resulting average CV (coefficient of variation) of 15%. The pH value of all test samples was maintained at 7 to prevent the pH-effect, before toxicity was measured. The microorganism

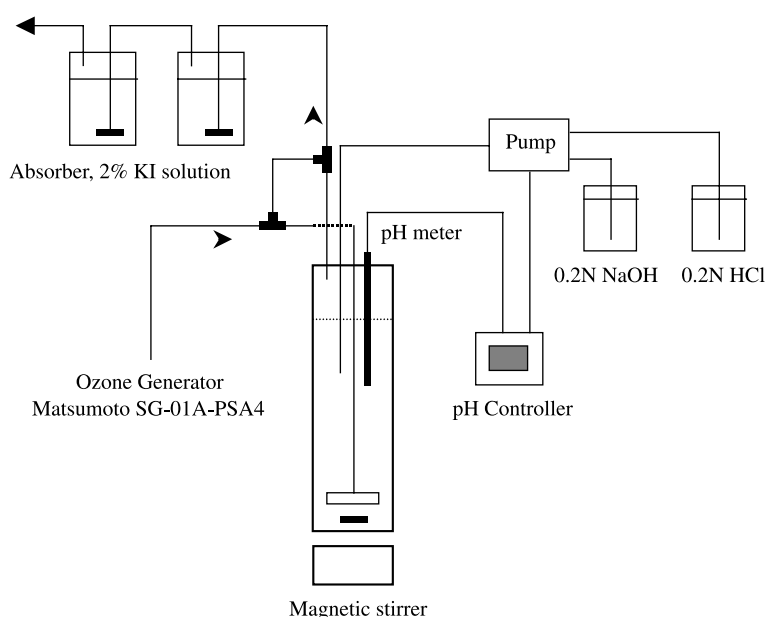


Fig. 1. Assemblage of ozonation system.

Table 1  
Operating conditions of ozonation

Gas phase	
Flow rate, $q$	976 mL/min
Pressure	1 kg/cm <sup>2</sup>
Influent ozone concentration, $[O_3]_{in}$	77.0 mg/L
Ozone dosage applied, $q \times [O_3]_{in}$	75.2 mg/min (1.57 mmol/min)
Liquid phase	
Bubble column reactor	327 mm high, 70 mm diameter
Volume of solution	1000 mL
Initial CPs concentration	100 mg/L (0.78 mM)
200 mg/L (1.56 mM)	
PH	7.0 ± 0.1

used in the Microtox produces light with peak intensity at 490 nm. Consequently, highly colored aqueous samples may cause nonspecific reductions in light level when analyzed according to the standard Microtox assay procedure. Any light level reduction could not be distinguished from one caused by toxicants. Therefore, a color correction procedure was incorporated in this study. For the Microtox test it is required to complete the measurement within 48 h after sampling, and the samples must be stored in a 4 °C environment. The EC<sub>50</sub>-values expressed as percentage (%) were used in this study. In addition, EC<sub>50</sub> values were transformed to toxicity units (TU) according to the approach introduced by Brown (1968). A toxicity unit is defined as follows:  $TU_i = C_i/EC_{50,i}$ , where  $C_i$  is the concentration of component  $i$ . Based on this, the TU values of the samples were determined by dividing TOC (mg/L) by the Microtox EC<sub>50</sub> value (%) as follows Eq. (1):

$$TU = TOC(mg/L)/EC_{50}(\%) \quad (1)$$

### 2.3. Oxygen Uptake Rate inhibition test

The microbial seed used for the Oxygen Uptake Rate (OUR) inhibition test was the activated sludge taken from a domestic wastewater treatment plant. The working sludge was not run through an acclimation process, and this sludge could be used within 6 h after sludge samples were collected from the treatment plant.

A dissolved oxygen (DO) meter with an electronic stirrer (YSI 5000) was used for OUR determinations. The mixed liquid suspended solid (MLSS) concentration was 217 mg/L during the whole OUR test, and the working volume was the standard BOD bottle of 300 mL. The organic carbon sources from glucose (non-inhibitory material) or test samples were fed at 50 mg/L of COD concentration, and the nutrient substrates were applied as 1 mL of solution A and B, described later. In this study, the OUR of glucose was the control value for defining the percentage of inhibition, which was determined as follows Eq. (2):

$$\begin{aligned} \text{Inhibition\%} &= [(OUR_{\text{samples}} - OUR_{\text{glucose}})/OUR_{\text{glucose}}] \\ &\times 100 = [1 - (OUR_{\text{samples}}/OUR_{\text{glucose}})] \times 100 \end{aligned} \quad (2)$$

The nutrient substrates used during the OUR test were as follows (Talinli and Tokta, 1994): solution A contained the following quantities per liter: K<sub>2</sub>HPO<sub>4</sub>, 320 g; KH<sub>2</sub>PO<sub>4</sub>, 160 g; NH<sub>4</sub>Cl, 120 g. Solution B contained the following quantities per liter: MgSO<sub>4</sub>·7H<sub>2</sub>O, 15 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; MnSO<sub>4</sub>·3H<sub>2</sub>O, 0.5 g; CaCl<sub>2</sub>, 2 g.

The OUR test was measured over a period of 15 min. During the measurement of OUR, the sludge was kept suspended with an electronic stirrer attached to a DO meter. The average OUR in each vessel was determined by plotting time versus DO concentrations. In this study, the specific OUR (SOUR) value was calculated as follows (Eq. (3)):

$$SOUR = OUR/MLSS = (\text{mg } O_2/\text{h})/\text{g MLSS} \quad (3)$$

## 3. Results and discussion

### 3.1. Decomposition and mineralization rates of chlorophenols by ozonation

Previous studies have indicated that the decomposition and mineralization rates of chlorophenols (CPs) by ozone treatment followed pseudo-first order kinetics:  $-dC/dt = kC$ , where  $C$  is the concentration of TOC (mg/L) or chlorophenols (mg/L), and  $k$  is the pseudo-first order rate constant (time<sup>-1</sup>) (Kuo, 1999; Shen et al., 1995). In this study, the pseudo-first order rate constant was determined by plotting reaction time versus the  $\ln(C/C_0)$  values of CPs or TOC concentrations. Figs. 2 and 3 illustrate the curves of decomposition and mineralization of CPs by ozone treatment at pH 7 and ozone dosage of 75.2 mg/min. The curves were found to be well fitted to the pseudo-first order kinetics, and the  $R$  square values were more than 0.93.

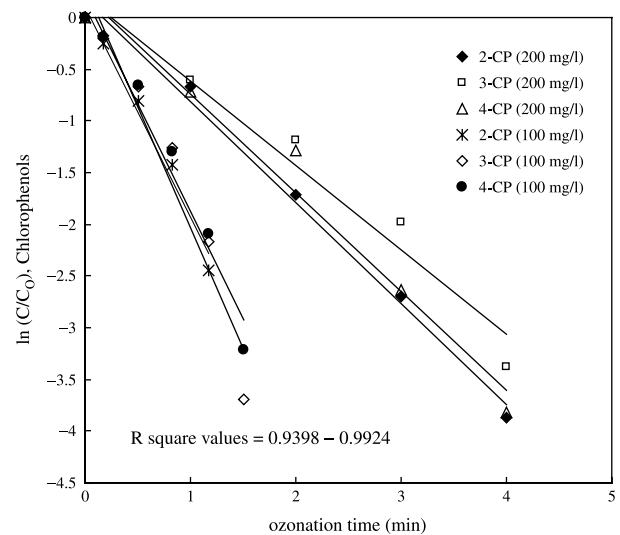


Fig. 2. The decomposition of chlorophenols (initial conc. 100 and 200 mg/L) by ozone treatment under pH 7 solution, ozone dosage applied = 75.2 mg/min.

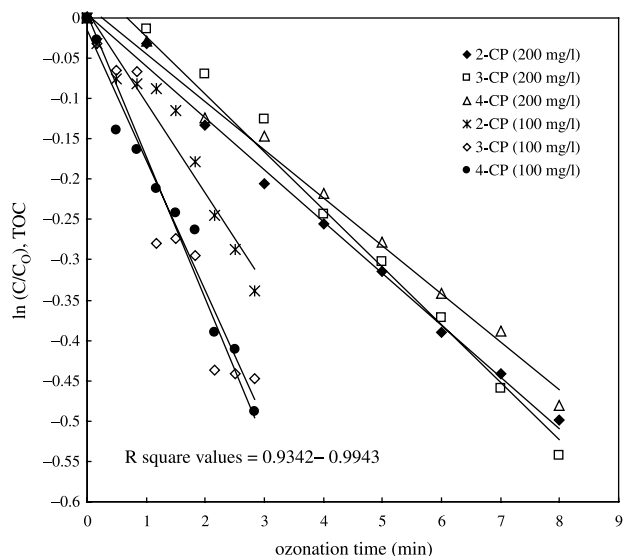


Fig. 3. The decomposition of TOC during ozonation of chlorophenols (initial conc. 100 and 200 mg/L) under pH 7 solution, ozone dosage applied = 75.2 mg/min.

Therefore, the slopes of the linear curves are the pseudo-first order rate constants and are summarized in Table 2, in which  $k_{CPs}$  and  $k_{TOC}$  are the pseudo-first order rate constants of decomposition of CPs and mineralization by ozonation, respectively. Comparison of the  $k_{CPs}$  and  $k_{TOC}$  values showed only slightly different results in the ozonation of 2-CP, 3-CP and 4-CP. Therefore, the o, m, and p-substitutes do not significantly affect the decomposition and mineralization rates of CPs by ozonation under neutral conditions.

### 3.2. Variation of toxicity during ozonation of chlorophenols

#### 3.2.1. Microtox toxicity and OUR inhibition test

The Microtox toxicity and Oxygen Uptake Rate (OUR) inhibition tests were conducted to monitor the variation of toxicity. The Microtox assay used the *P. phosphoreum* to determine the influence of toxic substances on pure bacteria. The OUR inhibition test utilized mixed bacteria from unacclimated activated sludge to evaluate the phenomenon of inhibition by ozonation byproducts.

Fig. 4 compares the variation of specific OUR values and Microtox EC<sub>50</sub> (%) during ozonation of 2-CP at an initial concentration of 200 mg/L and pH 7. The results revealed that the Specific OUR value of unozonated 2-CP was higher

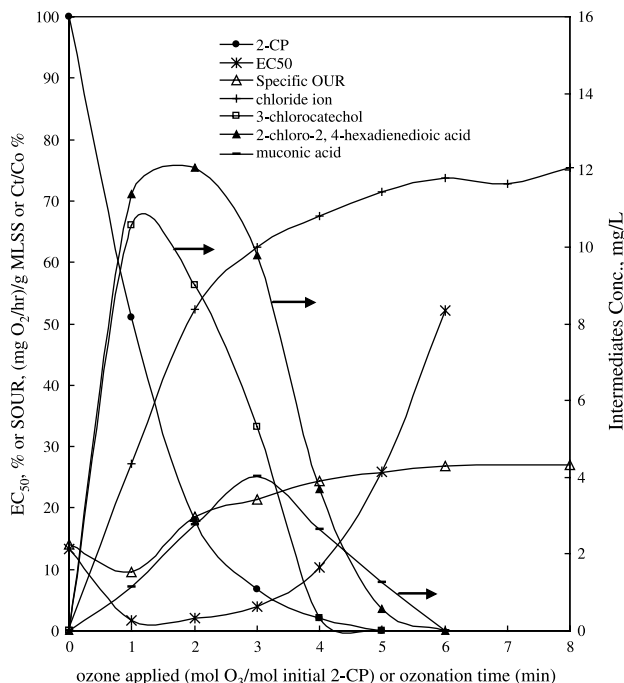


Fig. 4. The variation of 2-CP, intermediates and chloride during ozonation of 2-chlorophenol (initial conc. 200 mg/L) in pH 7 condition.

than that of the ozonated solution after 1 min of reaction time (when the ozone applied was 1 mol O<sub>3</sub> per mol of initial 2-CP). Since the lower SOUR value implies more inhibition to bacteria, ozonation of 2-CP significantly induced new aqueous toxicity to mixed microorganisms. Further ozonation could gradually increase the SOUR value after 2 min of reaction. The curve of the EC<sub>50</sub> (%) value showed a similar trend, the unozonated 2-CP solution having a higher value than the ozonated solution within 4 min of reaction time. The ozonated 2-CP also significantly induced new aqueous toxicity to pure bacteria in the Microtox toxicity. Some results of the Microtox test of ozonated 2-CP have been described in our previous study (Shang et al., 2002; 2003). Summarizing these results, the ozonated solution significantly induced new aqueous toxicity to the microorganisms. The oxidized 2-CP intermediates produced more serious toxicity to pure bacteria or mixed microorganisms than the parent compounds during the early stage of ozonation. The amount of ozone consumed that led to the maximum toxicity or inhibition was one mol per mol of initial 2-CP.

Based on the results of the OUR test, the percentage of OUR inhibition was calculated by Eq. (2) and is shown in Fig. 5. The percentage of inhibition is negative if the OUR value of the test sample is less than the control value (in this study the OUR of glucose was the control value). Contrarily, the inhibition is positive when the OUR<sub>samples</sub> is more than OUR<sub>glucose</sub>. The results revealed that the largest inhibition of OUR appeared at 1 min of ozonation time, and the percentage of inhibition was -63.8%. Further ozonation after 1 min of reaction could gradually decrease the degree

Table 2  
Decomposition and mineralization rates of chlorophenols by ozonation in pH 7 solution and ozone dosage applied 75.2 mg/min condition

Concentration (mg/L)	2-CP		3-CP		4-CP	
	100	200	100	200	100	200
$k_{CPs}^a$	2.04	0.977	2.338	0.815	2.085	0.955
$k_{TOC}^b$	0.112	0.064	0.177	0.072	0.162	0.060

<sup>a</sup> Pseudo-first order rate constant on the basis of CPs removal, min<sup>-1</sup>.  
<sup>b</sup> Pseudo-first order rate constant on the basis of TOC removal, min<sup>-2</sup>.

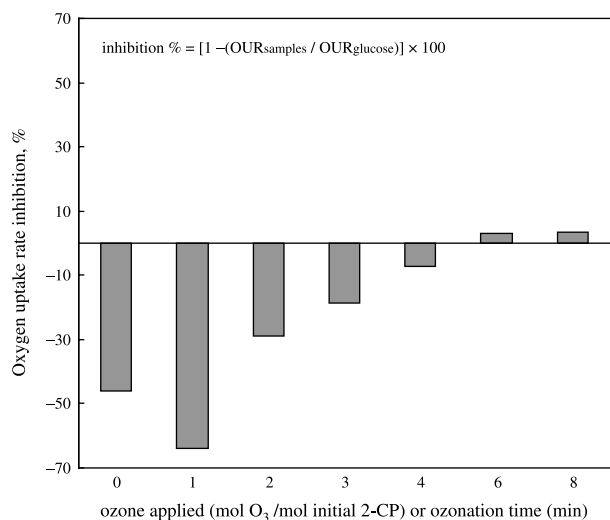


Fig. 5. Results of the OUR inhibition during ozonation of 2-CP (initial conc. 200 mg/L) in pH 7 condition.

of inhibition. However, the percentage of inhibition was negative until 6 min of oxidation, meaning the ozonated solutions became more biodegradable than glucose after 6 min of reaction.

Fig. 6 summarizes the results of the Microtox TU values during ozonation of 2-CP, 3-CP and 4-CP at pH 7. The TU value increased with time until a maximum value was reached. After the maximum point, the TU value decreased gradually with further oxidation and eventually became nontoxic. This result revealed that ozonation of 2-CP, 3-CP and 4-CP significantly induced new aqueous toxicity, and the increasing toxicity normally reached a peak in the early stage of ozonation. Some discussion of the Microtox test for ozonated chlorophenols is presented in our previously study (Shang et al., 2002; 2003). In summary, the intermediates of oxidized chlorophenols might produce the risk of acute

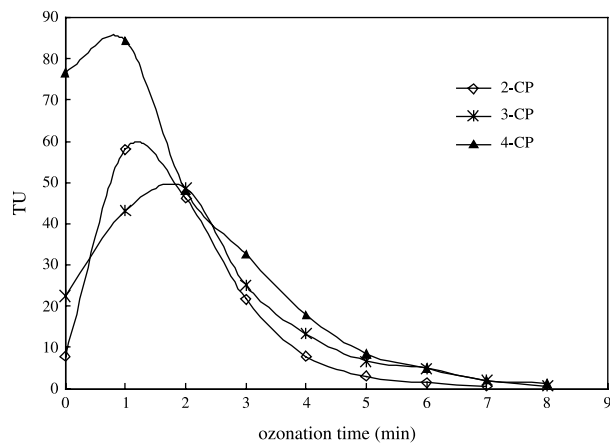


Fig. 6. The comparison of toxicity unit (TU) during ozonation of 2-CP, 3-CP and 4-CP under pH 7 solution,  $\text{TU} = \text{TOC (mg/L)} / \text{EC}_{50} (\%)$ .

toxicity to pure or mixed microorganisms, if these toxic intermediates are not further oxidized.

### 3.3. Relation between toxicity and ozonated intermediates

Fig. 4 illustrates the concentration of reaction products with ozonation time for the 2-CP solution. According to the HPLC analysis, 3-chloro-catechol and 2-chloro-2, 4-hexadienedioic acid were the major byproducts within 3 min of ozonation. The hydroxylated intermediate of 3-chloro-catechol was formed by electrophilic aromatic substitution of the hydroxyl group at the ortho-position. 2-chloro-2, 4-hexadienedioic acid was the next-step oxidant of 3-chloro-catechol through cleavage of the aromatic ring. Another study also indicated similar mechanisms in ozonation of 2-CP (Kuo and Huang, 1995). In addition, 3-chloro-catechol might be the precursor compound of the dimmer structure formed through the mechanism of oxidative coupling. The dimmer compounds are shown in Fig. 7. (Shang et al., 2002; Hirvonen et al., 2000). Hirvonen et al. (2000) indicated that the hydroxylated or chlorinated dimeric intermediates (chlorodihydroxybiphenols and dichlorodihydroxybiphenols) were formed during ozonation of 2-CP and 4-CP in acidic solution, and these dimmer compounds suggested that the two benzene rings were combined through C–C bonding. In our previous study, a compound of molecular weight 252 was detected by mass spectrometry in ozonation of 2-CP, which suggests that the dimmer compounds could be formed. Comparing the variation of toxicity and hydroxylated/chlorinated intermediates formed, we found that the maximum toxicity or inhibition to microorganisms occurred during the reaction period when these compounds formed in ozonated 2-CP. Base on this finding, 3-chloro-catechol, 2-chloro-2, 4-hexadienedioic acid and the dimmer compound may be well related to the sources of toxicity during the early stage of ozonation of 2-CP.

According to the HPLC analysis in this study, ozonated 3-CP and 4-CP formed chloro-catechols and chloro-muconic acids as the major intermediates after preliminary ozonation (Fig. 7) (Kuo and Huang, 1995). The intermediates of 4-chloro-catechol and 3-chloro-2, 4-hexadienedioic acid were the major byproducts during the ozonation of 4-CP. 4-chloro-catechol was formed by electrophilic aromatic substitution of the hydroxyl group at the ortho-position during the ozonation of 4-CP. 3-chloro-2, 4-hexadienedioic acid was the next-step oxidant of 3-chloro-catechol. However, two kinds of chloro-catechols transformed into chloro-muconic acids were observed in the ozonation of 3-CP. The hydroxylated byproducts of chloro-catechols were formed by electrophilic aromatic substitution in the ortho-position for both sides of the hydroxyl group on the benzene ring. Therefore, two kinds of chloro-muconic acids could be detected. In addition, our previous paper has reported the formation of high molecular weight compounds in ozonated 2-CP, 3-CP and 4-CP based on mass

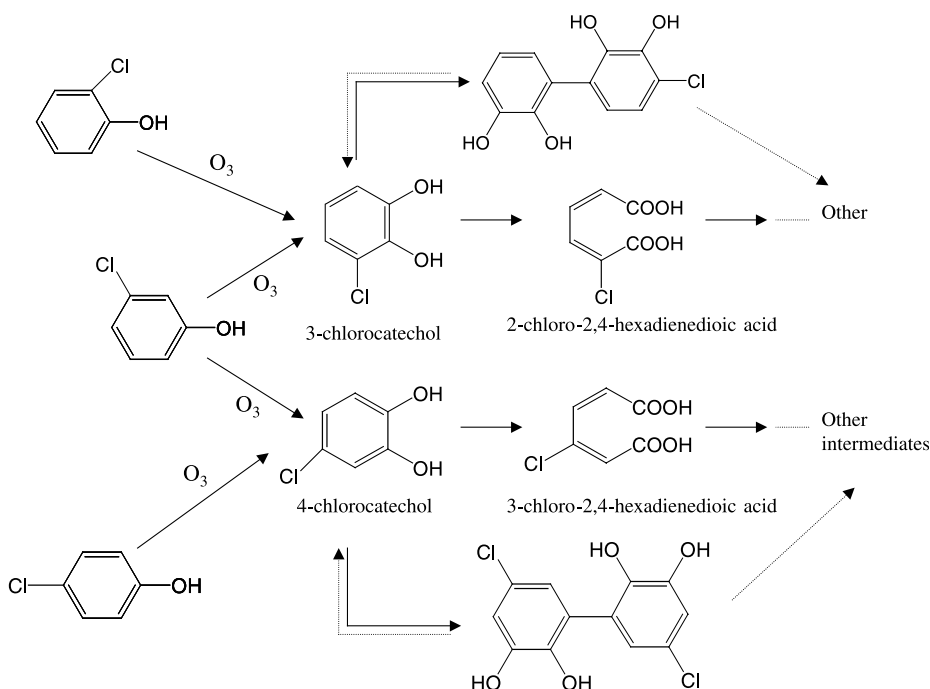


Fig. 7. Formation of hydroxylated/chlorinated dimeric compounds, chloro-catechols and chloro-muconic acids during ozonation of 2-CP, 3-CP and 4-CP.

spectrometry. (Shang et al., 2002). These high molecular weight compounds contain a hydroxylated/chlorinated substituent on the benzene ring, and the structures corresponding to the detected molecular weight are suggested in Fig. 7. Nevertheless, this study still could not indicate very clearly which of the above intermediates caused the increasing toxicity and inhibition of microorganisms.

#### 4. Conclusions

The results of this study revealed that the decomposition of 2-CP, 3-CP and 4-CP and the mineralization rates of TOC followed pseudo-first order kinetics during ozonation. The oxidized 2-CP solution induced new toxicity during the early stage of ozonation, as shown from Microtox  $EC_{50}$  and OUR inhibition tests. The results of the Microtox and OUR inhibition tests exhibited very similar trends in this study; the highest toxicity and the largest percentage of inhibition both occurred at 1 mol of applied ozone dosage per mol of initial 2-CP. Comparing the variation of toxicity and the hydroxylated/chlorinated intermediates formed, 3-chlorocatechol, 2-chloro-2, 4-hexadienedioic acid and the dimmer compound may be related to the sources of toxicity to pure bacteria and mixed microorganisms during the early stage of ozonation of 2-CP. In addition, the results revealed that ozonation of 2-CP, 3-CP and 4-CP significantly induced new aqueous toxicity if these toxic intermediates were not further ozonated.

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