The effect of Cu^{2+} chelation on the direct photolysis of oxytetracycline: A study assisted by spectroscopy analysis and DFT calculation[☆]

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ABSTRACT

The extensive usage of OTC and Cu^{2+} in livestock and poultry industry caused high residues in natural environment. Co-contamination of OTC and Cu^{2+} was a considerable environmental problem in surface waters. In this study, Cu^{2+} mediated direct photolysis of OTC was studied. Cu^{2+} chelating with OTC was found to greatly inhibit OTC photodegradation. To reveal the chelation mechanism of OTC-Cu complexes, multiple methods including UV–Vis absorption spectra, Infrared (IR) spectra, mass spectroscopy, and density functional theoretical (DFT) modeling were performed. Four OTC-Cu complexes were proposed. Cu^{2+} preferably bond to $\text{O}_{11}\text{—O}_{12}$ site with the binding constants $\log K = 8.19$ and 7.86 for CuHL^+ and $\text{CuL}^{\pm}$, respectively. The second chelating site was suggested to be $\text{O}_2\text{—O}_3$ with the binding constants of $\log K = 4.41$ and 4.62 for Cu_2HL_3^+ and Cu_2L_2^+ , respectively. The suppressed quantum yield of OTC by Cu^{2+} chelation was accused for their intra-/inter-molecular electron transfer, by which the energy in activated states was distributed. The occurrence of electron transfer between BCD ring and A ring also from BCD ring to Cu was evidenced by the TD-DFT result only for the OTC-Cu complexes. Besides, the cyclic voltammetry measurement also suggested one OTC-Cu(II)/OTC-Cu(I) redox couple. These results suggested that the persistence of OTC in environmental surface waters will probably be underestimated for neglecting the chelating effect of Cu^{2+} . The photolysis quantum yield of OTC-Cu complexes, as well as the specific molar absorption constants, the equilibrium binding constants of Cu^{2+} with OTC could contribute to more accurate kinetic models of OTC.

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1. Introduction

Oxytetracycline (OTC) which belongs to the family of tetracyclines (TCs) is a broad-spectrum antibiotic. It is widely used in human medicines and veterinary drugs for therapeutic purpose, and applied as the feed additive for promoting animal growth in livestock industry and fish farm (Halling-Sørensen et al., 1998;

Sarmah et al., 2006). It has already been known that most of the intakes of TCs antibiotics are excreted out of body in active forms (Daghrir and Drogui, 2013; Pan et al., 2011), and further are released into wider environmental components through manure dispersal, sewage irrigation, field runoff and so on (Halling-Sørensen et al., 1998). The concentration level of TCs in farm waste is up to ppm level (Pan et al., 2011), and in surface water was detected to be several tens of micro-gram per liter with high detection frequency (Jiang et al., 2011). Meanwhile, copper is also commonly applied as the growth promoter in swine diets (Jacela et al., 2010; NRC, 2012). The suggested dietary dosage of copper is up to 100–250 mg/kg (Jacela et al., 2010). Cooper concentrations in pig feces were

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approximately 5-times greater than in pig feeds (Li et al., 2007). The extensive usage of TCs and copper made their residues accumulate in natural environment. Therefore, the co-existence of OTC and copper will cause considerable co-contamination in the form of complexation, provided that OTC has multiple O- and N-containing moieties.

Various metal cations like Ca^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , Pb^{2+} , and Fe^{3+} can chelate with the poly-hydroxyl and carbonyl groups of TCs (Benesch et al., 1969; Wang et al., 2015; Werner et al., 2006; Zhao et al., 2013). The properties of TCs-metal ion-chelates have been investigated by multiple techniques, such as UV–Vis absorption spectroscopy and fluorescence spectroscopy (Carlotti et al., 2012), infrared (IR) spectroscopy (Zhang et al., 2012), circular dichroism spectroscopy (Jezowska-Bojczuk et al., 1993; Wessels et al., 1998), electronic analytical methods (Zhao et al., 2013), and nuclear magnetic resonance spectroscopy (Williamson and Everett, 1975). The binding sites and stoichiometry of the complexes were metal ion- and pH-dependent. However, the binding sites of TCs-Cu complexes were inconsistently concluded (Jezowska-Bojczuk et al., 1993; Zhang et al., 2012; Zhao et al., 2013). Lately, there were attempts of investigating the TCs-metal ion-chelates by the way of theoretical computation, for example the DFT/TD-DFT method (Amat et al., 2012; Marcial et al., 2009). Therefore the DFT/TD-DFT calculation can be applied on OTC-Cu system to help to elucidate the microscopic complexation mechanism and affiliation property.

The presence of copper has been shown to affect the environmental fate of TCs by complexation. Coexistence of tetracycline (TTC) and Cu^{2+} influences the mobility of TTC on soil components (Wang et al., 2008; Zhao et al., 2013). And Cu^{2+} could catalyze TTC hydrolysis in the presence of oxygen (Chen and Huang, 2008). Notably, recent examples have already touched the evidence that photoexcited TTC in combination with Cu^{2+} enhanced the protein fragmentation process (Khan and Musarrat, 2002), and combination of OTC and Cu had a synergistic negative influence on functional diversity of the soil microbial community (Kong et al., 2006). Direct photolysis is an important elimination pathway of TCs in surface waters (Chen et al., 2008; Pouliquen et al., 2007). However, to the best of our knowledge, there has been no report on the influence of copper on OTC photolysis as yet. Understanding the Cu^{2+} mediated environmental fate of antibiotic helps to well evaluate the environmental risk of antibiotics. Therefore, this study aims to (1) investigate the chelation mechanism of OTC- Cu^{2+} complexes through spectroscopy analysis and theoretical calculation; (2) elucidate the influence of Cu^{2+} chelation on the direct photolysis of OTC.

2. Materials and methods

2.1. Direct photolysis of OTC with & without Cu^{2+}

The photolysis kinetics of OTC was studied in the absence and presence of Cu^{2+} on a photochemical reactor of XPA-1 (Xujiang Electromechanical Plant, Nanjing, China). A 100 W mercury lamp was used as the light source. Its actual luminous intensity at 365 nm, 297 nm and 254 nm was measured to be 1500 $\mu\text{W}/\text{cm}^2$, 840 $\mu\text{W}/\text{cm}^2$, and 730 $\mu\text{W}/\text{cm}^2$, individually. A glass filter, ZWB2, was used to cut off the light below 300 nm and above 400 nm. The relative luminous intensity distribution of the mercury lamp was provided in the supplementary material (Fig. S1). As for the reason to choose the mercury lamp (300 nm–400 nm) as the light source which is higher than the UV and lower than the major part of sunlight (>400 nm), it is because the main purpose of this study is to understand the photolysis fate of OTC and OTC-Cu complexes in natural surface waters. Therefore, a UV lamp is not appropriate for this purpose because sunlight has little spectrum distribution

under 300 nm. OTC has two main absorption bands, ~250 nm and ~350 nm, which are contributed by different chromophoric moieties (discussed below). For the consideration whether OTC follows different photolysis pathways by a UV lamp or a mercury lamp, a monochrome UV lamp (254 nm) was also tested, but the data was not presented in this study. Briefly, the photolysis rate constant for OTC at pH 6.1 by the UV was 0.00223 min^{-1} , and for the OTC/ Cu^{2+} = 2/1 system was 0.00143 min^{-1} , and the HPLC graph showed the same photolysis products between the UV (254 nm) treatment and the mercury lamp (300–400 nm) treatment. On the other hand, A xenon lamp which is known to well model the sunlight spectrum seems very appropriate for the study purpose. However, OTC and OTC-Cu complexes has the main absorption spectra under 400 nm. The mercury lamp with the ZWB2 filter perfectly covered the bathochromic absorption band of OTC (~365 nm). Comparing to a xenon lamp, a mercury lamp would be more efficient to degrade OTC. Otherwise, the irradiation above 400 nm of a xenon lamp only produces heat. The water cooling system of the photochemical reactor was not adequate to cool down a xenon lamp. Moreover, one of the aims of the study is to obtain the photolysis quantum yield of OTC and OTC-Cu complexes. Theoretically, the photolysis quantum yield is independent of the light source, either a mercury lamp or a xenon lamp will not affect the photolysis quantum yield. Based on the above reasons, the mercury lamp (300 nm–400 nm) was chosen as the light source.

Oxytetracycline (ACROS Organic, USA, chemical purity > 95%) solution for photolysis was prepared and filled into the quartz cuvette ($\Phi 26 \text{ mm} \times 182 \text{ mm}$) and placed around the mercury lamp. The reaction cuvette rotated around the lamp to get a homogeneous irradiation. To avoid OTC hydrolyzing by Cu^{2+} in the presence of oxygen (Chen and Huang, 2008), the photolysis kinetic was conducted in oxygen-free conditions. Dissolved oxygen in solution was removed by purging with N_2 (99.99%) at a consistent flow rate of 0.3 L/min before (30 min) and during the reaction. Details about solution-preparing and sample-analyzing were described in the supplementary material Text 1.

2.2. Quantum yield calculation

In order to reveal the intrinsic photochemical reactivity of OTC-Cu complexes, the quantum yield of direct photolysis was calculated according to equation $\phi_d = -k_{\text{comp}}/2.303 \cdot \sum(I_0 \lambda \cdot \epsilon \lambda) \cdot (S/V) \cdot l$, where $I_0 \lambda$ (Einstein $\text{cm}^{-2} \text{ s}^{-1}$) is the incident light intensity at wavelength λ . $\epsilon \lambda$ ($\text{L mole}^{-1} \text{ cm}^{-1}$) is the molar absorption coefficient; S (cm^2) is the exposed area; V (mL) is the volume of solution; l (cm) is the light path length. The detailed mathematical derivation was provided in supplementary material Text 2.

2.3. IR spectroscopy of OTC-Cu complex

The OTC-Cu solution of the ratio 1/1 was collected after the photolysis reaction, and stored in darkness at 4 °C. There were macroscopic particulates of claret color condensed in the solution after one week. The solid sample were collected by filtering through a PTFE filter (0.22 μm) and air-dried for infrared (IR) analysis. The fourier transform (FT) IR of the sample and OTC solid standard were diluted in KBr pellet and measured on a Nicolet Nexus 870 spectrophotometer in the range of 400–4000 cm^{-1} at room temperature with a spectral resolution of $\pm 4 \text{ cm}^{-1}$. The solid sample was also re-suspended into acetone. This acetone based sample was then analyzed by an ESI mass spectrometer (LCQ Fleet, ThermoFisher Scientific).

2.4. UV–Vis spectroscopy of OTC as a function of Cu^{2+} ratio

The photolysis rate of compound usually correlates to its photoabsorption efficiency. Therefore the UV–Vis spectra of OTC and OTC–Cu mixture were studied at two pH segments of pH 6 and pH 10. At pH 6, OTC mainly existed in the zwitter form $\text{H}_2\text{L}^\pm$. At pH 10, OTC mainly existed in the form of HL^- . A series of OTC– Cu^{2+} mixtures with different concentration ratios were prepared in Milli-Q water. The concentration of OTC was equally at 50 μM . The pH of the OTC–Cu mixtures was recorded after mixing. The UV–Vis absorption spectra of the OTC–Cu mixtures were measured by a UV–2450 spectrometer (SHIMADZU, Japan).

2.5. Fluorescence spectroscopy of OTC as a function of Cu^{2+} ratio

The fluorescence spectroscopy reflects the non-productive energy dissipation process of the compound in activated states. OTC solutions mixed with Cu^{2+} with different concentration ratio were studied for fluorescence emission at four fixed pH conditions as 2.0, 5.5, 7.4 and 9.0. Introducing Cu^{2+} will change the aqueous pH value and pH significantly affects the fluorescence emission of OTC. Therefore, the pH of the mixtures was fixed to the initial value by HCl or NaOH. The fluorescence spectrum was measured on a Cary Eclipse Fluorescence Spectrophotometer (Agilent, USA) with the exciting wavelength of 365 nm.

2.6. Cyclic voltammetry (CV) measurement of OTC/ Cu^{2+} combination

CV was applied to investigate the feasibility of electron transfer of OTC and OTC–Cu complexes. OTC and OTC–Cu mixture (1/1, mole/mole), both at 500 μM and pH 5.5, were prepared in Milli-Q water according to the description in the photolysis part. The high concentration aimed to obtain a significant current signal. After deoxygenating, CV was performed on a CHI660D electrochemical workstation (Shanghai Chenhua Co., Ltd., China) at a scan rate 50 mV/s. A glassy carbon electrode (CHI 104, d = 3 mm) was used as the working electrode. A saturated calomel electrode (SCE) and a platinum wire electrode were used as the reference and auxiliary electrodes, respectively.

2.7. DFT study

The theoretical computation was performed on the Gaussian 03 software (Frisch et al., 2004). It aimed to correlate the chromophore of OTC species and OTC–Cu complexes to the absorption band, and to provide explicit insight into the electronic transition by photo excitation. OTC has four species H_3L^+ , $\text{H}_2\text{L}^\pm$, HL^- , and L^{2-} (Qiang and Adams, 2004). H_3L^+ is the fully protonated species of OTC as shown in Fig. 1. $\text{H}_2\text{L}^\pm$ derived from the deprotonation of the tricarbonyl group (OH_3) at pH around 3.3. The proton at the phenolic-diketone group (OH_{12}) and the dimethylamino group (NH_{4a}) further released to form species HL^- and L^{2-} , respectively, at pH 7–9. The four OTC species and two OTC–Cu complexes which would probably exist under neutral pH conditions, CuHL^+ and $\text{Cu}_2\text{HL}^{3+}$, were assigned for theoretical computation. The crystal structure of $\text{H}_2\text{L}^\pm$ was drew from Cambridge Structural Database (Allen, 2002; Groom et al., 2016). Based on $\text{H}_2\text{L}^\pm$, the other molecular structures were derived by associating/dissociating the H^+ or Cu^{2+} in the certain position. Remarkably, single Cu^{2+} ion was assigned onto OTC molecule through bidentate form (Fig. 1, discussed below). The geometric optimization of different OTC species and OTC–Cu complexes was fulfilled at the hybrid (U)B3LYP/6-31G(d) level by destiny functional theory (DFT) method (Hohenberg and Kohn, 1964). This method has already been successfully applied for the

calculation of TTC and CMT-3 (Amat et al., 2012; Marcial et al., 2009). It was notable that the distinguished basis set of LanL2DZ was appropriately selected for Cu (Hay and Wadt, 1985). The bulk solvent effect was estimated in water by the integral equation formalism polarized continuum model (IEFPCM) (Tomasi et al., 2005). Vibrational analysis was consecutively performed to check the absence of virtual frequencies and the stability of molecular structure. Based on the optimized geometry, the electron transition of each species in water was performed using the time-dependent (TD) approach (Stratmann et al., 1998) with the same function/basis set.

3. Results

3.1. Photolytic kinetics of OTC with & without Cu^{2+}

Fig. 2 shows the direct photolysis kinetics of OTC and OTC–Cu systems. Both were fitted well into the first-order rate kinetic. The apparent photolysis kinetic rate constants were provided in Table 1. The presence of Cu^{2+} greatly inhibited OTC photodegradation. Increasing Cu^{2+} concentration further decreased the photolysis rate of OTC. The pH of the OTC–Cu mixture reduced from 5.7 to 4.6 after introducing Cu^{2+} . It has already known that the photolysis rate of OTC decreased as pH is reduced (Shaojun et al., 2008). The kinetic rates of OTC–Cu systems were still much lower than the pure OTC system of pH 3.9. Therefore, it was not pH, but Cu^{2+} the key factor of controlling the significant inhibition effect of OTC photolysis. The quantum yields of OTC–Cu complexes were calculated and presented in Table 1. Comparing to $\text{H}_2\text{L}^\pm$ which is mainly existed in neutral pH, $\phi_d(\text{CuHL}^+)$ and $\phi_d(\text{Cu}_2\text{HL}^{3+})$ were much lower.

3.2. Cu^{2+} chelation affected the UV–Vis spectrum of OTC

The UV–Vis absorption spectrum of OTC greatly shifted in the presence of Cu^{2+} ions at both pH 6 and pH 10 segments (Fig. S2). Take pH 6 for example, from the OTC without Cu^{2+} to the OTC/ Cu^{2+} ratio of 1/2, the bathochromic band underwent a wide red shift of 20 nm, the absorption of the hypsochromic band grew up, meanwhile the pH reduced from 6.1 to 4.6. In the overlapped absorption spectra two isosbestic points appeared at 361 nm and 303 nm in this range. From the OTC/ Cu^{2+} ratio of 1/2 to that of 1/20, the red-shift of the bathochromic band stopped, and a slight blue-shift occurred to the hypsochromic band. The pH slightly decreased from 4.6 to 4.3. The isosbestic points shifted to 345 nm and 280 nm, respectively.

Four OTC–Cu complexes: CuHL^+ , $\text{Cu}_2\text{HL}^{3+}$, $\text{CuL}^\pm$, and Cu_2L^{2+} were proposed in this study (discussed below). The molar absorption constants of the four complexes were resolved by global analysis of the Cu^{2+} mediated absorption spectra (Fig. S2), using the software ReactLab EQUILBRIA (Jplus Consulting). The results were provided in Fig. 3. By this approach, the equilibrium binding constants of Cu^{2+} association to OTC was derived and provided in Table 2. The values were close to the previous reports (Carlotti et al., 2012; Couto and Reis, 2000; Jezowska-Bojczuk et al., 1993; Zhao et al., 2013). It indicated that the global fitting approach was reliable for calculating the complexation affinity. The specific molar absorption constants of complexes and the equilibrium binding constants of Cu^{2+} with OTC were subsequently used for calculating the photolysis quantum yield.

3.3. Cu^{2+} chelation inhibited the fluorescence spectra of OTC

The fluorescence spectrum of OTC–Cu complexes under different OTC/Cu ratio at pH 2.0, pH 5.5, pH 7.4 and pH 9.0 were

Fig. 1. Molecular structure of oxytetracycline, and the three-dimensional geometries of HL^- , L^{2-} and $\text{Cu}_2\text{HL}^{3+}$.

Fig. 2. Direct photolysis kinetic of OTC and OTC-Cu mixture. $[\text{OTC}]_0 = 80 \mu\text{M}$.

Fig. 3. The molar absorption coefficients of OTC-Cu complexes.

Table 1
Photolysis kinetic and quantum yield of OTC species, CuHL^+ , and $\text{Cu}_2\text{HL}^{3+}$.

Species	$ K_{\text{app}} /\text{min}^{-1}$	$\tau_{1/2}/\text{min}$	R^2	H_3L^{+b}	$\text{H}_2\text{L}^{\pm b}$	HL^{-b}	CuHL^+	$\text{Cu}_2\text{HL}^{3+}$
pH = 3.9	0.0171 ± 0.0002	41	0.9991	17.4%	82.6%			
pH = 5.7	0.0201 ± 0.0002	35	0.9991	0.3%	98%	1.7%		
2:1, pH 5.0	0.0106 ± 0.0001	65	0.9989	0.9%	52.3%	0.2%	44.9%	1.7%
1:1, pH 4.7	0.00796 ± 0.0001	100	0.9977	0.8%	23.4%		62.5%	14.3%
1:2, pH 4.6	0.00482 ± 0.0001	144	0.9993	0.1%	3.8%		43.1%	53.0%
$K_{\text{comp}}/10^{-4} \text{ s}^{-1}$				>0.6	3.3	1.7	0.4	1.3
$\Sigma(I_{\lambda} \cdot \epsilon_{\lambda})/s^{-1}$				0.106	0.121	0.129	0.132	0.133
$\phi_d/10^{-4}$				>2.5	12	5.7	1.3	4.2

^a The proportion of each component was calculated by Visual MINTEQ 3.1.

^b Quantum yields of OTC species were from our previous study (not yet published).

provided in Fig. S3. The presence of Cu^{2+} intensively quenched the fluorescence emission of OTC. The quenching effect reached to the maximum limit when adding double amount of Cu^{2+} concentration

than OTC. The fluorescence emission of OTC greatly enhanced with increasing pH from 2.0 to 9.0. The fluorescence intensity of L^{2-} was estimated to be 10- to 30-fold stronger than the other protonated

Table 2
Equilibrium binding constant of Cu^{2+} associating to OTC.

Equilibrium	LogK	Cite
$\text{Cu}^{2+} + \text{HL}^- \rightleftharpoons \text{CuHL}^+$	8.19 ± 0.01^a	9.55 (Jezowska-Bojczuk et al., 1993) 7.61 (Couto and Reis, 2000) 7.06 (Zhao et al., 2013)
$\text{Cu}^{2+} + \text{CuHL}^+ \rightleftharpoons \text{Cu}_2\text{HL}^{3+}$	4.41 ± 0.02^a	3.60 (Carlotti et al., 2012)
$\text{Cu}^{2+} + \text{L}^{2-} \rightleftharpoons \text{CuL}^{\pm}$	7.86 ± 0.11^b	12.44 (Jezowska-Bojczuk et al., 1993) 10.49 (Couto and Reis, 2000) 10.06 (Zhao et al., 2013)
$\text{Cu}^{2+} + \text{CuL}^{\pm} \rightleftharpoons \text{Cu}_2\text{L}^{2+}$	4.62 ± 0.05^b	

^a Data derived from global fitting of the UV–Vis absorption spectra of Fig. S2a.

^b Data derived from global fitting of the UV–Vis absorption spectra of Fig. S2b.

OTC species.

3.4. IR spectroscopy of OTC-Cu complex

The solid sample from the OTC-Cu 1/1 system after photolysis gave the mass-to-charge ratio of 523 (Fig. S4). The mass spectroscopy result clearly suggested one OTC-Cu complex CuHL^+ which was combined by one Cu^{2+} ($m = 64$) plus one HL^- ($m = 459$) molecule. IR spectroscopy analysis of OTC and the OTC-Cu complex was provided in Fig. 4. The IR spectrum of OTC-Cu was different than that of OTC in several points: the absorption peak at 3604 cm^{-1} which reflects the stretching vibration of Ph-H disappeared; stretching vibration of O–H and N–H at 3487 cm^{-1} and 3371 cm^{-1} shifted to short wavelength; stretching vibration of C=O indicated by 1653 cm^{-1} , 1618 cm^{-1} , 1589 cm^{-1} showed varied specific absorption in OTC-Cu complex. The IR spectra variation suggested Cu^{2+} probably bind with OTC through C=O at C_{11} and C_{2a} . Complexation at $\text{C}_{10}\text{--O}$, $\text{C}_{12}\text{--O}$, and N_{4a} could also be possible.

3.5. CV measurement of OTC-Cu complex

CV measurement provided an insight into the property of inter/intramolecular electron transfer of OTC and OTC-Cu complexes. Fig. 5 shows the CV results that no reaction occurred on the electrode in pure OTC solution, while the OTC-Cu solution appeared one oxidation wave and two reduction waves. After calibrating to standard hydrogen electrode (SHE), the anodic peak potential was 180 mV (E_{pa}) with the peak current $2.10 \text{ } \mu\text{A}$ (i_{pa}); The potentials of the two cathodic peaks were -117 mV (E_{pc1}) and -470 mV (E_{pc2}),

Fig. 5. Cyclic voltammetry measurement of OTC and OTC-Cu mixture (ratio of 1/1) in aqueous solution. Scan rate: 50 mV/s .

with the corresponding peak current $1.86 \text{ } \mu\text{A}$ (i_{pc1}) and $5.75 \text{ } \mu\text{A}$ (i_{pc2}). The ratio of the anodic peak current to the cathodic peak current at -117 mV , $i_{\text{pa}}/i_{\text{pc1}}$, equals to 1.1. It indicated a quasi-reversible redox process which probably attributed to the redox couple OTC-Cu(II)/OTC-Cu(I). The formal potential of the possible OTC-Cu(II)/OTC-Cu(I) couple, $E_{1/2} = (E_{\text{pa}} + E_{\text{pc1}})/2$, is 82 mV (vs. SHE). However, it was unclear which reaction occurred at E_{pc2} .

3.6. Theoretical electronic absorption spectroscopy and the electron transition

By geometric optimization, species H_3L^+ , $\text{H}_2\text{L}^{\pm}$, and HL^- hold the twisted conformation (A) of which the dimethylamine group situates above the BCD plane to release the steric hindrance between the protonated NH_{4a} and OH_{12a} . Species L^{2-} holds the extended conformation (B) of which the dimethylamine group lies under the BCD plane (Fig. 1). The computed conformations were as same as stated by Wessels et al. (1998). The optimized special molecular geometry showed $\text{C}_{12}\text{--O}$ and $\text{C}_1\text{--O}$ were not stretching to the same plane, with the dihedral angle about 60° in both conformation A and B. Therefore, Cu^{2+} was not likely to chelate onto these two positions. The space distance between sites N_{4a} and O_{12a} is too far away ($4.96 \text{ \AA}$) in the twisted conformation, whereas too crowded in the extended conformation, to insert one Cu^{2+} ion.

Two OTC-Cu complexes were assigned for the theoretical calculation, CuHL^+ and $\text{Cu}_2\text{HL}^{3+}$, taking $\text{O}_{11}\text{--O}_{12}$ as the first binding site, and $\text{O}_{2a}\text{--O}_3$ as the second binding site. The mass spectroscopy of the OTC-Cu complex sample suggested one complex species of CuHL^+ , no aqua ligands or Cl^- was included onto Cu. Therefore,

Fig. 4. Infrared spectroscopy of OTC and OTC-Cu complex.

Cu^{2+} chelating onto OTC were assigned as the bidentate form. CuHL^+ of ground state was presumably assigned to be at doublet state because Cu^{2+} participated in the chelation with odd electrons. Due to the presence of two single electrons marked the unpaired electron spins, $\text{Cu}_2\text{HL}^{3+}$ of the ground state was assigned to be at triplet state.

As Fig. 6 shows, the TD-DFT method successfully calculated the UV–Vis absorption spectrum of OTC species. The peak of the long-wavelength absorption was calculated to be 333 nm (H_3L^+), 339 nm (H_2L^+), 354 nm (HL^-), and 375 nm (L^{2-}), which were in accordance with the experimental red shift of the bathochromic band. The calculated long-wavelength absorption of CuHL^+ and $\text{Cu}_2\text{HL}^{3+}$ reached 381 nm and 404 nm. However, the calculated absorption band of CuHL^+ and $\text{Cu}_2\text{HL}^{3+}$ were not appropriately located as experimental UV-spectra (Table S1).

The electronic transitions of the H_3L^+ , H_2L^+ , HL^- , L^{2-} and CuHL^+ , $\text{Cu}_2\text{HL}^{3+}$ were provided in supporting information (Table S1). The visualized molecule orbitals of the electron transition were shown in Fig. 7. From the three dimensional electron population, it is obvious that the BCD ring of OTC generated the absorption above 300 nm through $\pi \rightarrow \pi^*$ transition of the phenol ring (Ph) and the phenolic-diketone group (P–K). The tricarbonyl group (Tcm) only contributed the short-wavelength absorption at about 250 nm. The electron transition revealed the occurrence of intermolecular charge transfer of OTC–Cu complexes. Take CuHL^+ as the example, at 379 nm and 250 nm, the electron transfers from A ring to BCD ring by photo exciting; at 326 nm, the transition through HOMO-2 to LUMO+2 showed a 7% possibility of ligand-to-metal charge transfer (LMCT) from BCD ring to Cu. For $\text{Cu}_2\text{HL}^{3+}$, when Cu chelated to $\text{O}_{2a}\text{--O}_3$, the intermolecular charge transfer also processed from phenol ring to the Cu at A ring. By comparison, OTC species did not show a possibility of intermolecular charge transfer between A ring and BCD ring.

4. Discussion

4.1. Binding sites of Cu^{2+} with OTC

IR spectra did not provide robust evidence of the chelation sites, but suggested the carbonyl sites. Referring to the previous study about binding sites of TCs (Jezowska-Bojczuk et al., 1993; Zhang et al., 2012; Zhao et al., 2013), the possible binding sites of OTC for Cu^{2+} were $\text{N}_{4a}\text{--O}_{12a}$, $\text{O}_{10}\text{--O}_{11}$, $\text{O}_{11}\text{--O}_{12}$, $\text{O}_{12}\text{--O}_1$, and $\text{O}_{2a}\text{--O}_3$. The possibility of $\text{O}_{12}\text{--O}_1$ and $\text{N}_{4a}\text{--O}_{12a}$ sites has already been excluded as a result of the steric limitation. It was stated in Result part 3.6. TD-DFT results revealed that BCD ring contributed to the bathochromic absorption, and A ring contributed to the hypsochromic absorption. Therefore, the red shift of the bathochromic band induced by Cu^{2+} chelation at low concentration suggested Cu^{2+} firstly participated into BCD ring.

In the UV absorption study, pH decreased from 6.1 to 4.9 by adding 1/1 ratio Cu^{2+} (Fig. S2a), indicated Cu^{2+} competed with H^+ for the binding site. OH_3 is completely deprotonated above pH 5.5. The possibility of Cu^{2+} firstly binding to O_3 was excluded. It was reasonable to substitute the proton at O_{12} because the association constant of Cu^{2+} to HL^- was fitted to be 8.19 which is higher than $\text{p}K_2$ (7.46) (Qiang and Adams, 2004), while OH_{10} was regarded to have the $\text{p}K_4$ value up to 11.8 (Duarte et al., 1999). Cu^{2+} competed for the site of the Ph–OH (C_{10}) was unlikely to happen. Substituting H^+ from N_{4a}H site was not convincing because the association constant of Cu^{2+} to HL^- was lower than $\text{p}K_3$ (8.94) (Qiang and Adams, 2004). Therefore, it can be speculated that Cu^{2+} chooses $\text{O}_{11}\text{--O}_{12}$ as the preferable binding site. Then it was reasonable that the pH only decreased by 0.3 unit when introducing 1/1 ratio Cu^{2+} to the OTC solution at condition of pH 10.0 (Fig. S2b), in which condition OTC mainly existed as the form of L^{2-} . Taken together, the complex CuHL^+ was formed in neutral pH condition, and $\text{CuL}^{\pm}$ formed in basic condition.

A 1–2 ligand-to-metal species was proposed as $\text{Cu}_2\text{HL}^{3+}$ or Cu_2L^{2+} , when Cu^{2+} concentration increased to high. The second chelating site was suggested to be $\text{O}_{2a}\text{--O}_3$. It was evidenced by the UV absorption spectra at pH 6.1 (Fig. S2) that (1) the blue shift

Fig. 6. Theoretical UV–Vis absorption spectroscopy of OTC species comparing to the experimental ones.

also from ligand to Cu for OTC-Cu complexes, but not for OTC species. The CV measurement also gave the clue of the OTC-Cu(II)/OTC-Cu(I) couple performing reversible one-electron redox process. The formal potential of 82 mV equals 7.9 kJ/mol ($\Delta G = -nE_{1/2}F$) which is small and indicated such electron transfer process occurs freely.

5. Conclusion

Four OTC-Cu complexes (CuHL^+ , Cu2HL^{3+} , $\text{CuL}^\pm$, and Cu2L^{2+}) were proposed on the basis of UV–Vis absorption spectra, IR spectra and DFT modeling. Cu^{2+} chelated firstly to $\text{O}_{11}\text{--O}_{12}$ site, then O2--O3 site as the second binding site when Cu^{2+} concentration increases. The high associating constants of OTC-Cu complexes indicated Cu^{2+} will strongly chelate to OTC in their co-existed system, and the two species of CuHL^+ and Cu2HL^{3+} will be the dominants in natural pH condition of containing Cu^{2+} . Parameters from this study like the equilibrium binding constants of Cu^{2+} with OTC, specific molar absorption constants and direct photolysis quantum yields of OTC-Cu complexes could serve for evaluating the environmental fate of OTC.

The presence of Cu^{2+} typically inhibited the direct photolysis rate of OTC in neutral pH condition. The visible electronic transition derived by TD-DFT calculation, as well as the oxidation/reduction peaks from the CV measurement, concluded the inter/intramolecular electron transfer of OTC-Cu complexes resulted in the inhibition effect. The influence of Cu^{2+} on OTC photolysis overthrows the common preconception that a red-shift of absorption spectrum will induce an enhancement of photodegradation. It disagreed with OTC-Cu complexes. Cu^{2+} chelation inhibited the photolysis activity of OTC, resulting in increased persistence of OTC in natural surface waters. Therefore, it is necessary to re-cognize the Cu^{2+} chelation effect for more accurate and better understanding the environmental fate and risk of antibiotics in conditions of containing Cu^{2+} .

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2016.04.084>.

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