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Photodegradation of the azole fungicide fluconazole in aqueous solution under UV-254: Kinetics, mechanistic investigations and toxicity evaluation

Zhi-Feng Chen, Guang-Guo Ying, Yu-Xia Jiang, Bin Yang, Hua-Jie Lai, You-Sheng Liu, Chang-Gui Pan, Fu-Qiang Peng*

State Key Laboratory of Organic Geochemistry, CAS Centre for Pearl River Delta Environmental Pollution and Control Research, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

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ABSTRACT

The azole fungicide fluconazole has been reported to be persistent in conventional wastewater treatment plants. This study investigated the photodegradation of fluconazole under UV-254 in aqueous solutions. The results revealed that the photodegradation of fluconazole was pH-dependent (2.0–12.0) following the pseudo-first-order kinetics with quantum yield values ranging from 0.023 to 0.090 mol einstein^{-1}, and it underwent a direct and self-sensitized mechanism involving $^1O_2$. The main photodegradation by-products were identified and semi-quantitated. The proposed photodegradation pathway included hydroxylative defluorination reaction. The 72 h-NOEC and 72 h-LOEC values for fluconazole using a freshwater unicellular green alga *Pseudokirchneriella subcapitata* were 10 μM and 15 μM. Overall, the photodegradation of fluconazole produced a significant decrease in algal toxicity. It also proved that the photodegradation by-products will not present extra toxicity to this alga than fluconazole itself.

1. Introduction

Fluconazole is an azole fungicide that is widely used as active ingredient in a variety of pharmaceutical and personal care products (PPCPs) to treat fungal infections by blocking the sterol biosynthesis (Zarn et al., 2003). Fluconazole has a water solubility of approximately 1.39 g/L (Wishart et al., 2008), and log K_{ow} and vapor pressure values of 0.25–0.4 and 3.89×10^{-7} Pa, respectively (Garcia-Valcarcel and Tadeo, 2012), indicating its hydrophilic nature. After application, fluconazole enters into wastewater treatment plants (WWTPs), and then reaches the receiving environment. Fluconazole has been reported in influent, effluent and surface water at concentration ranges of 22–570 ng/L, 28–140 ng/L and 1–111 ng/L, respectively (Chen et al., 2012; Kahle et al., 2008; Kim et al., 2009; Lindberg et al., 2010; Peng et al., 2012). It was found to have low removal rates in conventional WWTPs (Kahle et al.,

* *Corresponding author*. Tel./fax: +86 20 85290200.
E-mail addresses: guangguo.ying@gmail.com, guang-guo.ying@gig.ac.cn (G.-G. Ying).

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2008). Thus the chemical fluconazole in the aquatic environment may cause unpredicted adverse effects on nontarget organisms. In fact, it has been demonstrated that fluconazole is a CYP3A4 inhibitor and also a potent CYP2C9 inhibitor (Wishart et al., 2008). In addition to its inhibition of cytochrome P450s, fluconazole has been implied to have endocrine disrupting activities (Kjaerstad et al., 2010). Therefore, the azole fungicide fluconazole has attracted an increasing attention due to its persistence and potential biological effects.

It has been demonstrated that fluconazole is very resistant to microbial degradation under conventional activated sludge treatment (Kahle et al., 2008). Advanced oxidation technologies may be an alternative option for the elimination of fluconazole in wastewater streams. Photolysis under UV irradiation is a process which is quite effective for some chemicals in the advanced treatment for wastewater or drinking water (Hijnen et al., 2006; Kang et al., 2004; Legrini et al., 1993). UV-254 uses a low-pressure mercury lamp emitting at 254 nm, as the most common light source in the photodegradation process. In fact, UV-254 photodegradation has been investigated for the removal of some emerging contaminants such as PPCPs (Benitez et al., 2013; Canonica et al., 2008; Liu et al., 2011; Marotta et al., 2013). In addition, reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-\$}$) and even stronger oxidant hydroxyl radical (•OH) can be photogenerated by certain PPCPs (Agrawal et al., 2007; Chen et al., 2008; Ge et al., 2010; Inbaraj et al., 2002), indicating that photodegradation could be a promising technology for the removal of emerging contaminants like fluconazole. However, there has been no photodegradation report available for fluconazole. Therefore, it is necessary to clarify the photodegradation behavior of fluconazole and associated toxicity change during its photolysis process in order to minimize any potential risks.

The objectives of this study were to determine the rate constants and the quantum yields for the photodegradation of fluconazole in different pH solutions under UV-254, and to elucidate the role of the ROS in the photolysis. Moreover, the photodegradation by-products were identified and semi-quantitated to explain the photodegradation mechanism. The toxicity change during the photodegradation of fluconazole was also assessed by algae toxicity testing.

2. Experimental section

2.1. Chemicals and reagents

Fluconazole (FZ, 99.0%), atrazine (AZ, 97.4%) and deuterium oxide (D_2O, 99.9%) were obtained from Dr. Ehrenstorfer, Riedel-de Haen and J&K Chemical, respectively. Reagents of HPLC grade (methanol, acetonitrile, ethyl acetate and dichloromethane) were purchased from Merck and CNW Technologies. HPLC grade water was obtained from a Milli-Q water purification system (Millipore). Oasis HLB cartridges (500 mg, 6 mL) were supplied by Waters. The other chemicals and reagents were of analytical grade. Stock solutions of fluconazole and atrazine were prepared in Milli-Q water at concentrations of 1000 mg L^{-1} and 100 mg L^{-1}, respectively. The freshwater unicellular crescent-shaped green alga *Pseudokirchneriella subcapitata* for toxicity testing was obtained from the Adelaide Laboratory of the Commonwealth Scientific and Industrial Research Organization (CSIRO, Australia).

2.2. Photodegradation experiments

2.2.1. Photodegradation kinetics

Photodegradation experiments for fluconazole were carried out in a home-made photo-reactor (Fig. S1) with a 10 W low-pressure mercury (LP Hg) lamp ($\lambda = 254$ nm) (Beijing Cel Scitech Co., Ltd., China). The LP Hg lamp as UV irradiation source is inserted into the center of a cylinder reactor with Pyrex outer walls. The light is filtered through a circulatory water jacket situated between the source and the reaction vessel, which controls the temperature of the solution. The circulatory water jacket is made of quartz to let 254 nm radiation reach the solution, and the reaction solution is maintained at 21 ± 1 °C.

A working solution of fluconazole (2 μM, 400 mL) was prepared in water containing 5 mM phosphate (monopotassium phosphate and dipotassium phosphate) for all kinetic experiments. Dark controls were included in the experiments, and no loss of fluconazole was observed in the dark. The kinetic experiments were conducted in duplicate.

The effect of pH (2.0, 2.8, 5.0, 7.0, 9.0, 11.0 and 12.0) on the photodegradation kinetics was tested in 5 mM phosphate buffer solution. Hydrochloric acid and sodium hydroxide were employed for pH adjustment. The accurate pH values were determined using a Thermo Orin 5 star pH meter (Thermo Fisher Scientific, USA).

The effect of oxygen (O_2) was examined in normal state solution (reaction solution contains O_2 at pH 7.0) and N_2 saturated solution at pH 7.0. The roles of ROS were examined using deuterium oxide (D_2O, 70 mM) (Scharffetter-Kochanek et al., 1993) and tertiary butanol (tBuOH, 500 μM). D_2O was used to prolong the lifetime of $^1O_2$ in order to promote the oxidation involving $^1O_2$ (Wilkinson et al., 1995; Zhang et al., 2010). tBuOH was employed as triplet state scavenges in addition to being a scavenger of •OH which may be generated from $O_2^{-\$}$ during a series of reactions (Benitez et al., 2013; Chen et al., 2008; Li et al., 2011). A control test was performed using D_2O (70 mM) and tBuOH (500 μM) in the N_2 saturated solution to demonstrate that D_2O or tBuOH does not compete with the target compound for the light energy. All the D_2O and tBuOH experiments were conducted in 5 mM phosphate buffer solution (pH = 7.0).

The concentrations of fluconazole were determined using an Agilent 1200 series high performance liquid chromatograph (HPLC, Agilent, USA) system equipped with a diode array detector. The detailed instrumental conditions are described in Supplementary Information (Text S1). The limit of quantification for fluconazole and atrazine was 0.04 μM and 0.03 μM, respectively. For all kinetic experiments tested under different conditions, the photodegradation of fluconazole was fitted with the pseudo-first-order kinetic equation (Eq (1)):

$$-\ln(C_t/C_o) = k \times t \quad (1)$$

$$DT50 = (\ln 2)/k \quad (2)$$

Where C_o and C_t are the fluconazole concentrations at the initial time and reaction time t, respectively; k is the pseudo-first-order constant and DT50 is the photodegradation half-life.

2.2.2. Identification and semi-quantitation of photodegradation by-products

For photodegradation by-products identification, another photodegradation experiment was performed at a higher initial concentration of 3.3 μM for fluconazole than that used in the kinetic experiment. The reaction solution (400 mL) of fluconazole was photo-reacted at pH 7.0 and 21 ± 1 °C. At the time of 0, 1, 2, 3 and 5 h, every 400 mL reaction solution was transferred into a 1 L amber bottle and then adjusted to pH 3.0. A control experiment with the blank solution without the addition of fluconazole was also conducted. Solid phase extraction (SPE) was used for extracting the photodegradation by-products from the reaction solutions. The reaction solutions were loaded at a flow rate of 5 mL min^{-1} on the SPE cartridges (Waters Oasis HLB, 500 mg, 6 mL), which had been previously preconditioned each with 2 × 5 mL methanol and 2 × 5 mL Milli-Q water. Then the cartridges were dried under vacuum for 3 h. Residual fluconazole and its photodegradation by-products were eluted with 3 × 2 mL dichloromethane, 3 × 2 mL ethyl acetate and 3 × 2 mL methanol in sequence. These three eluates from each cartridge were combined, then dried under a gentle nitrogen stream and finally redissolved in 1 mL methanol into a 2 mL amber glass vial (Anpel, Shanghai, China) before UHPLC-MS/MS analysis.

For semi-quantitation of photodegradation by-products, the solutions from the kinetic experiments under the conditions of pH 2.0, pH 7.0, pH 12.0, N_2 aeration, in the presence of D_2O and in the presence of tBuOH were also detected by UHPLC-MS/MS.

Photodegradation by-products were identified and semi-quantitated by an Agilent 1200 series ultra high performance liquid chromatograph (Agilent, USA) coupled to an Agilent 6460 triple quadrupole mass spectrometer with electrospray ionization mode (UHPLC-ESI-MS/MS). The detailed instrumental conditions are given in Supplementary Information (Text S2). The mass spectrometer was operated under both positive and negative ionization mode for the identification and only under positive ionization mode for the semi-quantitation. For preliminary screening of by-products, MS^2 scan mode was conducted at a fragmentor voltage of 135 eV with mass scan range of 50–1000 amu. For confirmation of by-products, product ion mode was used following the conditions in Table S1. For semi-quantitation of by-products, multiple reaction monitoring (MRM) mode was performed following the conditions in Table S2.

2.2.3. Quantum yield estimation at 254 nm

Quantum yield was determined by chemical actinometry for weakly absorbing systems (Zepp, 1978) using 3 μM aqueous atrazine as an actinometer (solution buffered at pH = 7.0 with 5 mM phosphate) with a quantum yield of 0.047 mol $einstein^{-1}$ (Hessler et al., 1993) and molar absorption coefficient of 3860 M^{-1} cm^{-1} at a wavelength of 254 nm (Nick et al., 1992). For the same photo-reaction system (reactor, light, solution and volume), the quantum yield (Φ) for the photo-reaction of fluconazole can be calculated according to eq (3) (Zepp, 1978):

$$\Phi_{FZ} = ((k_{FZ} \times \varepsilon_{AZ})/(k_{AZ} \times \varepsilon_{FZ})) \times \Phi_{AZ} \quad (3)$$

where k_{FZ} and k_{AZ} are the photodegradation rate constants for fluconazole and atrazine, respectively; ε_{AZ} and ε_{FZ} are the molar absorption coefficients of atrazine and fluconazole; and Φ_{AZ} is the quantum yield for the photo-reaction of atrazine, which was used as a reference compound. The HPLC conditions to determine the concentrations of atrazine are described in Text S1. The determination of the molar absorption coefficients (ε) of fluconazole at the wavelength of 254 nm under different pH conditions was also carried out using a Lambda 850 spectrophotometer (PerkinElmer, USA). For detailed experiment information, please refer to Supplementary Information (Text S3).

2.3. Toxicity evaluation

2.3.1. Sample preparation for toxicity

Owing to the lack of aquatic toxicity data for the azole fungicide fluconazole, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values of fluconazole were obtained using a freshwater unicellular green alga *Pseudokirchneriella subcapitata* according to previously reported testing methods (Lewis et al., 1994; Yang et al., 2008). This green alga was also used to test toxicity changes during the photodegradation of fluconazole. The photodegradation experiments were performed in Milli-Q water at pH 7.0 and 21 ± 1 °C under UV-254 irradiation in the photoreactor. Considering its NOEC value of 10 μM, a high initial concentration of 20 μM for fluconazole was chosen for toxicity evaluation. At the time of 0, 1, 3, 5 and 9 h, the solutions (30 mL each time) were collected for the algal toxicity test. Another 1 mL was used for the determination of residual fluconazole concentrations. Corresponding to the residual fluconazole concentrations at different stages of photodegradation, the same fluconazole concentrations without any photodegradation by-products were prepared as the control group by diluting the stock solution.

2.3.2. Calculation and statistical analysis

Growth inhibition tests of *P. subcapitata* over 72 h with fluconazole and its by-products followed the procedures described by Yang et al. (2008) with a little modification. Algal toxicity tests were carried out in 20 mL narrow-mouth flasks containing 10 mL of algal culture. Both control (positive and negative) and test flasks were inoculated with exponentially growing algae at an initial concentration of approximately 1.0 × 10^4 cells/mL. The cultures were incubated at 24 ± 2 °C under continuous illumination, then vortex mixed for three times in one day. Yield of algal cells was calculated indirectly by measuring the optical density of the cell suspension at a wavelength of 420 nm using a Lambda 850 spectrophotometer. A good linear relationship between growth of algal species and the absorbance of cell suspension was obtained as found in Yang et al. (2008). The absorbance of algae was determined at 72 h after the start of each test. Growth inhibition rate (GIR) was calculated according to Eq (4):

$$GIR = [1 - (A/\bar{A}_c)] \times 100\% \quad (4)$$

where A is the absorbance for the sample and $\bar{A}_c$ is the average of the absorbance for the control without any fluconazole or its by-products. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values of fluconazole were calculated using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test (Lewis et al., 1994) by the software SPSS 13.0. The basic assumptions of the Dunnett's Procedure, normality and homogeneity of variance are formally tested. Shapiro–Wilk's Test is used to test for normality, while the test for homogeneity of variance is the Bartlett's Test. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Tests, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test. The significance level was set at $p = 0.05$.

3. Results and discussion

3.1. Influence of pH on photodegradation of fluconazole

Fluconazole (FZ) was found to be stable in the dark controls, but was degraded under the UV-254 irradiation, demonstrating that hydrolysis is not a factor in fluconazole photodegradation. At pH 7.0, the removal percentage of fluconazole was 84% after UV-254 irradiation for 300 min. Meanwhile, the photodegradation of fluconazole followed the pseudo-first-order reaction kinetics ($R^2 > 0.99$, $p < 0.05$) with half-life (DT50) and quantum yield (Φ) of 115 ± 4 min and 0.023 ± 0.001 mol einstein^{-1}, respectively (Table S3).

According to the pK_a values (2.56, 2.94 and 11.01) of fluconazole with four forms in aqueous solution (Correa et al., 2012), the pH dependence of its photodegradation was investigated with pH values ranging from 2.0 to 12.0. Fig. 1 showed that the photodegradation rate constant (k) of fluconazole increased at pH < 7.0 or > 7.0, and followed an order: pH 12.0 > pH 2.0 > pH 2.8–11.0, indicating that alkaline condition is preferred to acidic and neutral conditions for the photodegradation of fluconazole. A similar trend is also observed for k, molar absorption coefficient (ε, calculated from Fig. S2) and Φ (The detailed information is reported in Table S3). Due to the distribution of the protonation states of fluconazole (FZ) (Correa et al., 2012) (Fig. S3), FZ^{2+}, FZ^{+}, FZ and FZ^{-} were the dominant species in solution at pH 2.0, pH 2.8, pH 7.0 and pH 12.0, respectively. Therefore, the photodegradation trend of fluconazole (FZ) follows the order: $FZ^{-} > FZ^{2+} > FZ^{+} > FZ$ (Φ, 0.090 > 0.039 > 0.030 > 0.023 mol einstein^{-1}). It should be noted that with pK_a values of 2.56 and 2.94, FZ^{+} may be the dominant species at pH 2.8 but it is certainly not the only one. This experiment confirms that the photodegradation of fluconazole under UV-254 is pH-dependent.

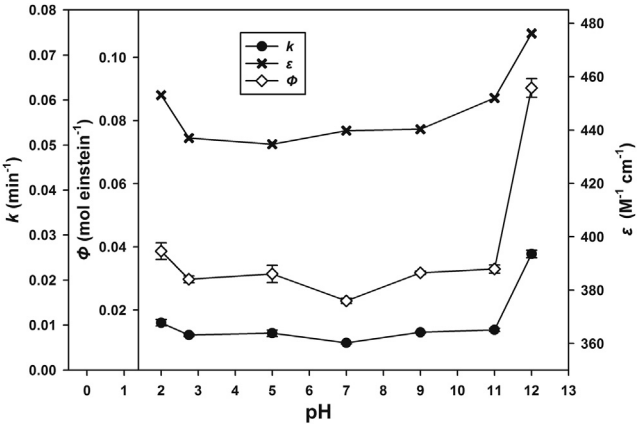


Fig. 1 – Photodegradation rate constant (k), molar absorption coefficient (ε) and quantum yield (Φ) as a function of pH for fluconazole (FZ) under UV-254 ($[FZ]_o = 2$ μM). The error bars represent standard deviations ($n = 2$).

3.2. Influence of ROS on photodegradation of fluconazole

The photodegradation of fluconazole investigated for the role of ROS in 5 mM phosphate buffer solution (pH = 7.0) followed pseudo-first-order reaction kinetics ($R^2 > 0.99$, $p < 0.05$). k value was markedly higher in the "with-O_2"solution (Normal) than in the "without-O_2" solution (N_2) (Fig. 2). The result suggests that O_2 plays an important role in the photodegradation of fluconazole by inducing the generation of ROS. Moreover, it also suggests that fluconazole undergoes both direct and self-sensitized photodegradation.

As shown in Fig. 2, no evident change in k value between N_2, $D_2O + N_2$ and tBuOH + N_2 indicated that D_2O or tBuOH did not compete with the target compound for the light energy. In

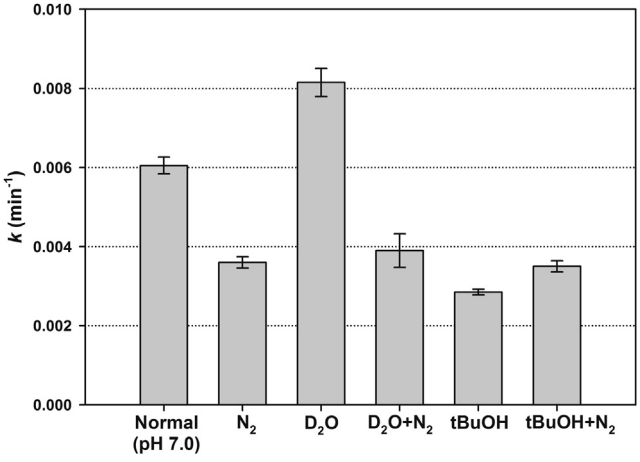


Fig. 2 – Photodegradation rate constant (k) of fluconazole (FZ) under different conditions. The conditions used in the experiment include: Normal (reaction solution contains oxygen, the same as the condition of pH 7.0), N_2 (nitrogen saturated solution makes the reaction solution out of oxygen), D_2O (in the presence of 70 mM D_2O), $D_2O + N_2$ (in the presence of 70 mM D_2O under nitrogen saturated solution), tBuOH (in the presence of 500 μM tBuOH) and tBuOH + N_2 (in the presence of 500 μM tBuOH under nitrogen saturated solution). All of the conditions were conducted under pH = 7.0 ($[FZ]_o = 2$ μM). The error bars represent standard deviations ($n = 2$).

the presence of D_2O and O_2, k value significantly increased compared to the condition of normal state (Fig. 2). The increased rate constant in the presence of 70 mM D_2O was probably due to the role of $^1O_2$ in the degradation process of fluconazole as D_2O was expected to prolong the lifetime of $^1O_2$. With regards to the effect of tBuOH, we could find that k value observed in the presence of tBuOH was lower than in the condition of normal state and in N_2 saturated solution (Fig. 2), implying that tBuOH may not only be a •OH quencher, but also a FZ* scavenger. Actually, alcohols can be triplet state or •OH scavengers, and they can also inhibit the photodegradation process (Vione et al., 2010). Therefore, the effect of tBuOH does not exclude, but it does not prove that •OH is involved in the degradation process. In this context, a simplified generation scheme of ROS (Eq (5)) and the effect of tBuOH (Eqs (6) and (7)) could be as follows:

$$FZ^* + {}^3O_2 \rightarrow FZ + {}^1O_2 \quad (5)$$

$$FZ^* + tBuOH \rightarrow FZ^{-\$} + tBuOH^{+\$} \quad (6)$$

$$FZ^{-\$} + {}^3O_2 \rightarrow FZ + O_2^{-\$} \quad (7)$$

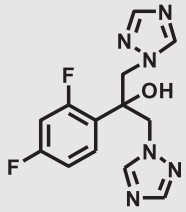
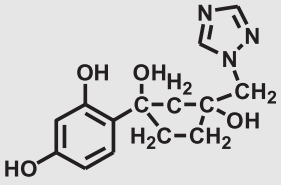
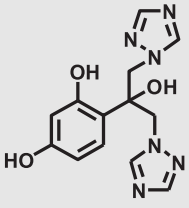
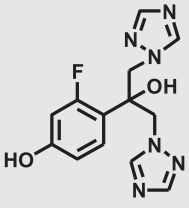
where FZ* is the excited state of fluconazole that is excited by UV-254; and $^3O_2$ is the ground state of oxygen.

3.3. *Photodegradation mechanism of fluconazole*

3.3.1. *Identification of photodegradation by-products*

Photodegradation of fluconazole produced a variety of by-products, as demonstrated by UHPLC-MS/MS analysis (Fig. S4). The chromatograms (both positive and negative ionization mode) presented in Fig. S4 clearly showed the

Table 1 – Main mass fragments of fluconazole (FZ) and its by-products obtained by UHPLC-ESI-MS/MS in both positive and negative mode.

Compound/retention time (min)	Positive mode $[M + H]^+$		Negative mode $[Mâ'H]^-$		Proposed structure
	m/z values (relative abundance)		*m/z* values (relative abundance)		
	MS^2 scan	Product ion[a]	MS^2 scan	Product ion[a]	
Fluconazole (FZ) 20.0	307 (100%) 238 (89%) 220 (83%) 169 (43%)	220 (100%) 238 (78%) 169 (72%) 70 (43%) 141 (11%)	305 (100%) 191 (99%) 222 (23%) 68 (2%)	191 (100%) 68 (76%) 108 (17%) 122 (16%) 222 (1%)	
A (FZ-CP) 5.6	292 (100%) 223 (5%)	223 (100%) 70 (74%) 109 (20%) 137 (10%) 81 (6%)	–	–	
B (FZ-2OH) 10.6	303 (100%) 147 (51%) 234 (46%) 216 (17%)	147 (100%) 70 (10%) 234 (3%) 137 (2%) 216 (1%)	301 (100%) 218 (8%) 214 (4%)	68 (100%) 214 (85%) 218 (38%) 163 (30%) 135 (17%)	
C (FZ-OH) 17.8	305 (100%) 149 (6%) 236 (5%)	149 (100%) 70 (25%) 236 (14%) 218 (2%) 139 (2%)	303 (100%) 220 (3%)	68 (100%) 220 (18%) 137 (16%) 165 (9%) 151 (4%)	

[a] The product ions were generated from the highest parent ions as shown in MS^2 scan, e.g. *m/z* 307 (100%) and 305 (100%) for FZ.

detected photodegradation by-products of fluconazole during the UV-254 irradiation. Three by-products from the photodegradation of fluconazole were identified using MS^2 scan mode for preliminary screening and using product ion scan mode for confirmation (Table 1). To elucidate the chemical structures of fluconazole by UHPLC-MS/MS, the MS^2 spectra obtained from the product ion scan mode of fluconazole and its photodegradation by-products provided the basis of interpretation (Figs. S5–S11). For instance, in positive mode, certain fragments indicated the presence of the intact triazolyl ring (Fig. S5, 70 *m/z* (1)) including 2-propanol group (Fig. S6, 126 *m/z* (6)) and 2-propanone group (Fig. S7, 124 *m/z* (3)). In reference to the functional group of aromatic ring, fragments 141 *m/z* (Fig. S5, (4)), 139 *m/z* (Fig. S8, (4)) and 137 *m/z* (Fig. S6, (7) and Fig. S7, (4)) were assigned to the phenyl with substitution of two fluorine atoms, a hydroxyl group and a fluorine atom, and two hydroxyl groups, respectively. The occurrence of the three fragments 53 *m/z* which originated

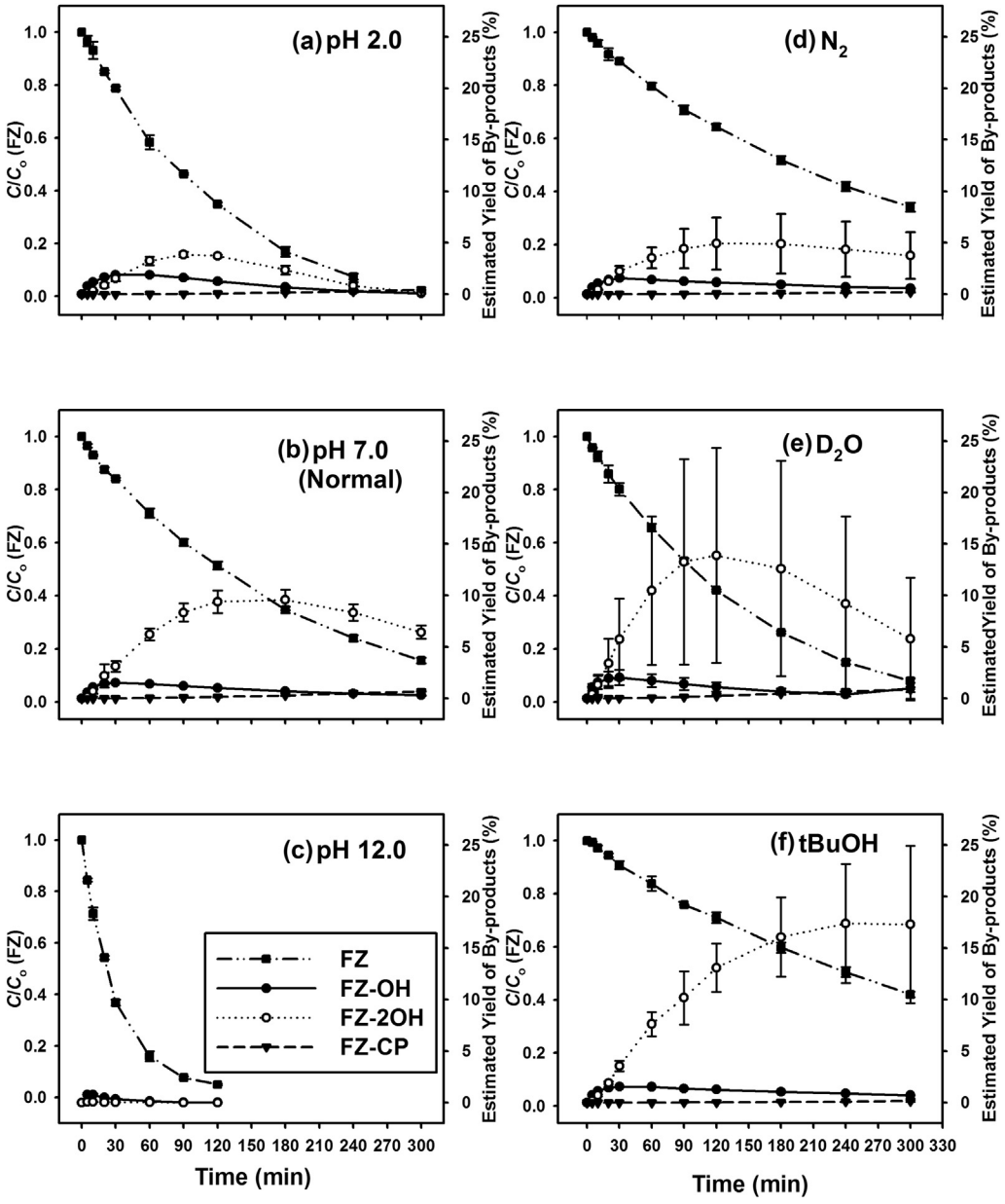


Fig. 3 – The presence rate of fluconazole (FZ) and the estimated yield of the photodegradation by-products (FZ-OH, FZ-2OH and FZ-CP) in different irradiation times under different condition: (a) pH 2.0, (b) pH 7.0 (reaction solution contains oxygen, the same as the condition of Normal), (c) pH 12.0, (d) N_2 (nitrogen aeration makes the reaction solution out of oxygen), (e) D_2O (in the presence of 70 mM D_2O) and (f) tBuOH (in the presence of 500 μM tBuOH) ($[FZ]_o$ = 2 μM). The error bars represent standard deviations (n = 2). FZ-OH: 2-(2-fluoro-4-hydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; FZ-2OH: 2-(2,4-dihydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; FZ-CP: 1-(2,4-dihydroxyphenyl)-3-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol.

from rearrangement of ring structures, 67 *m/z* and 81 *m/z* (Fig. S6, (1), (2) and (4)) suggested a fully intact pentyl ring. Fluconazole (FZ) was found at the retention time of 20.0 min with a molecular ion peak of *m/z* 307 and *m/z* 305 in the positive and negative mode, respectively. For the by-product **A** at the retention time of 5.6 min, the molecular ion peak (*m/z* 292) in the positive mode indicated 1-(2,4-dihydroxyphenyl)-3-(1H-1,2,4-triazol-1-ylmethyl)-1,3- cyclopentanediol (FZ-CP). The by-products **B** and **C** at the retention times of 10.6 and 17.8 min showed a similar mass spectrum to that of fluconazole, with a mass difference of 4 and 2 in their molecular ions and other fragment ions in both positive and negative mode. The substitution of fluorine atom for hydroxyl group would lead to the molecular ion peaks *m/z* 303 and 305 in positive mode and *m/z* 301 and 303 in negative mode, respectively. These two by-products **B** and **C** were assigned as 2-(2,4-dihydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol (FZ-2OH) and 2-(2-fluoro-4-hydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol (FZ-OH). These photodegradation by-products which were generated by hydroxylative defluorination were demonstrated in a previous study (Ge et al., 2010). For the by-product **C** (FZ-OH), hydroxyl group reacted preferably with 4-fluorine atom than 2-fluorine atom due to the steric-hindrance effect. Meanwhile, the substitution of hydroxyl group could increase the polarity of the chemical. It is in accordance with the polar tendency FZ-CP > FZ-2OH > FZ-OH > FZ as shown in Fig. S4.

3.3.2. *Semi-quantitation of photodegradation by-products*

Semi-quantitation of the by-products during the UV-254 irradiation was carried out in this study (Fig. 3). The estimated yield of by-products was calculated using the area of the by-product at different time versus the area of the initial fluconazole (0 h). As shown in Fig. 3, the by-products FZ-2OH (**B**) and FZ-OH (**C**) firstly increased to reach maximum values and then decreased. The time to reach the maximum values for FZ-OH was faster than for FZ-2OH, indicating that FZ-OH could transform to FZ-2OH, and then FZ-2OH would further transform to other by-products. FZ-CP (**A**) was found to slowly increase in all of the conditions, except for pH 12.0 possibly due to its instability in alkaline solution. Overall, acidic or neutral solution is beneficial for fluconazole to produce by-products **A**–**C** compared to alkaline solution (Fig. 3). Under both oxygenated water and anoxic water scenarios (Fig 3b; and Fig 3d), the three main by-products (FZ-OH, FZ-2OH and FZ-CP) were all detected during the degradation process, indicating that they can be generated in both direct and self-sensitized photodegradation. At the end of the photodegradation experiment (300 min), the estimated yields of FZ-OH, FZ-2OH and FZ-CP were 0.31%, 6.42% and 0.69% in oxygenated water, and 0.59%, 3.76% and 0.19% in anoxic water.

3.3.3. *Proposed degradation pathway*

Considering the product identification and the semi-quantitation results, a plausible degradation pathway of fluconazole is proposed in Fig. 4. First, fluconazole is excited to the excited state (FZ*) by UV-254. Then two routes are included in the proposed mechanism for the degradation of fluconazole. Via route (1) with direct photodegradation and route (2) with $^1O_2$, fluconazole (FZ) degrades to FZ-OH, FZ-2OH and FZ-CP. The generation of FZ-OH and FZ-2OH proves that the photodegradation of fluconazole is subject to hydroxylative defluorination which has been reported before (Ge et al., 2010). In addition, FZ-OH could be converted to FZ-2OH, then FZ-2OH may further be transformed to FZ-CP.

3.4. *Elimination of fluconazole toxicity by photodegradation*

3.4.1. *NOEC and LOEC*

The concentration levels of fluconazole were set at 0 (control), 2, 6, 10, 15, 20 and 50 μM ($n = 3$). No significant loss of fluconazole was found during 72 h exposure. Via Shapiro–Wilk's

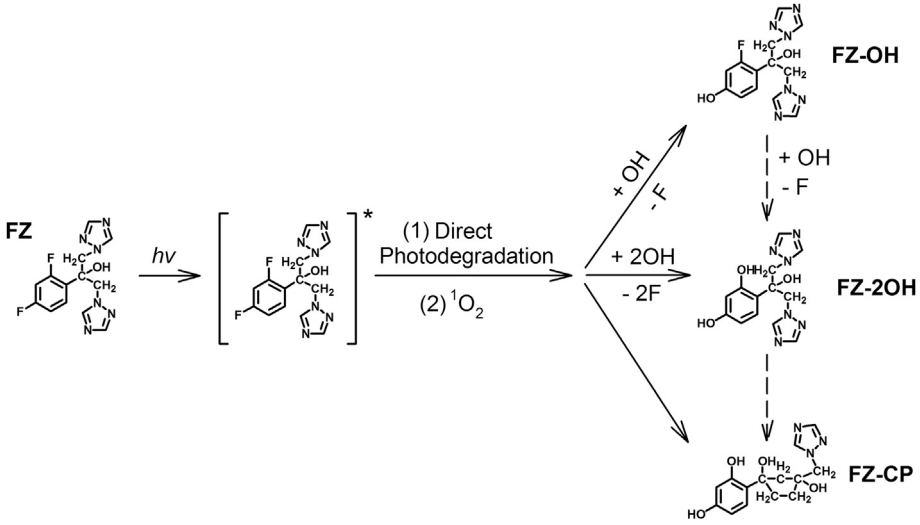


Fig. 4 – Proposed photodegradation by-products and pathways of fluconazole (FZ) under UV-254. FZ-OH: 2-(2-fluoro-4-hydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; FZ-2OH: 2-(2,4-dihydroxyphenyl)-1,3-bis(1H-1,2,4-triazol-1-yl) propan-2-ol; FZ-CP: 1-(2,4-dihydroxyphenyl)-3-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol.

Test, Bartlett's Test and Dunnett's Test, the NOEC and the LOEC after 72 h for fluconazole were 10 μM and 15 μM, respectively (Fig. S12). For this set of data, the minimum difference in mean absorbance between the control and any fluconazole concentration that can be detected as statistically significant is 0.0106. It is the first time that the toxicity of fluconazole to this aquatic organism is reported.

3.4.2. *Evaluation of toxicity change*

As shown in Fig. 5, no difference between the treated sample and the standard sample at 0 h meant that there was no external pollution for the photodegradation process. Meanwhile, the degradation curve for the treated samples (Fig. 5) shows a decline in fluconazole concentration with time because of photodegradation. Compared to the standard sample, the growth inhibition rate of the treated sample was similar at 1 h but gradually decreased from 3 h to 9 h (Fig. 5). This result clearly demonstrates that the degradation process of fluconazole under UV-254 led to loss of toxicity and that the by-products generated by photodegradation did not exhibit any appreciable degree of inhibitory effect relative to fluconazole itself. A growth promoting effect was found in the treated samples at 3, 5 and 9 h, suggesting that certain nutrients such as small nitrogen- and carbon-containing compounds were produced through the photodegradation of fluconazole.

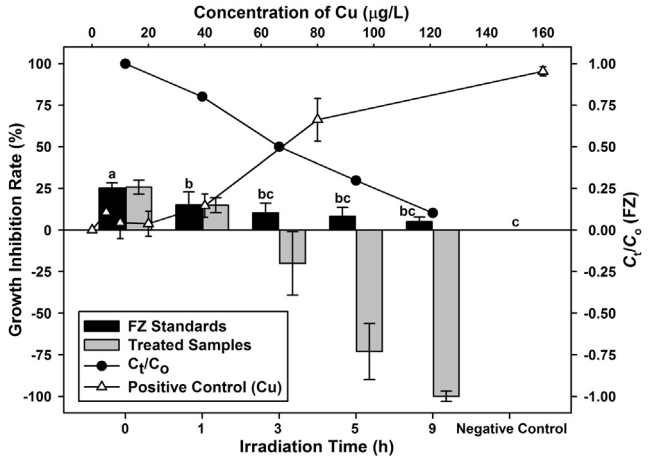


Fig. 5 – Toxicity evaluation obtained from *Pseudokirchneriella subcapitata* growth inhibition test for fluconazole (FZ) standard samples and treated samples, and the corresponding presence rate (C/C_o) of fluconazole under photodegradation in different irradiation times (pH = 7.0, $[FZ]_o$ = 20 μM). The standard samples diluted from the stock solution are the same fluconazole concentrations with the corresponding treated samples but without any photodegradation by-products. The treated samples were obtained from the photodegradation of fluconazole in different irradiation times. The error bars represent standard deviations (n = 3). Letters (a, b and c) indicate the significant difference of growth inhibition rate data by Student-Newman-Keuls test, $p < 0.05$. Growth inhibition rate data with letters a, b or c statistically differ from each other, while those with letters "b and bc", or "c and bc" suggest no significant variations.

4. Conclusion

This study demonstrated the photodegradation of fluconazole under UV-254, which followed pseudo-first-order kinetics, with rate constants and quantum yields varying according to the pH of the aqueous solution. According to the pK_a values, the photodegradation capacity of fluconazole follows the order: $FZ^- > FZ^{2+} > FZ^+ > FZ$ (Φ, 0.090 > 0.039 > 0.030 > 0.023 mol einstein^{-1}). With pK_a values of 2.56 and 2.94, it is notable that FZ^+ may be the dominant species at pH 2.8 but it is certainly not the only one. Meanwhile, fluconazole was photodegraded in the aqueous solution under UV-254 irradiation via a direct and a self-sensitized mechanism involving $^1O_2$. Based on the photodegradation by-products identified by UHPLC-MS/MS, a photodegradation mechanism of fluconazole is tentatively proposed, and involves hydroxylative defluorination reaction. Moreover, the algal toxicity testing demonstrated the loss of toxicity by photolysis, and no inhibitory effect of the photodegradation by-products relative to fluconazole itself. The results from this study can assist water industry and regulatory bodies in the control and risk management of this azole fungicide in wastewater and aquatic environments.

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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.watres.2013.12.039

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