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Biodegradation of typical azole fungicides in activated sludge under aerobic conditions

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ABSTRACT

Widespread use of azole fungicides and low removal efficiency in wastewater treatment plants (WWTPs) have led to the elevated concentration of azole fungicides in receiving environment. However, there was limited research about the removal mechanism of azole fungicides in the biological treatment of WWTPs. Imidazole fungicide climbazole and triazole fungicide fluconazole were selected to investigate the biodegradation mechanism of azole fungicides in activated sludge under aerobic conditions. Climbazole was found to be adsorbed to solid sludge and resulted in quick biodegradation. The degradation of climbazole in the aerobic activated sludge system was fitted well by the first-order kinetic model with a half-life of 5.3 days, while fluconazole tended to stay in liquid and had only about 30% of loss within 77 days incubation. Ten biotransformation products of climbazole were identified by high resolution mass spectrometry using suspect and non-target screening method. But no biodegradation products of fluconazole were identified due to its limited removal. The possible biodegradation pathways for climbazole were proposed based on the products identification and pathway prediction system, and involves oxidative dehalogenation, side chain oxidation and azole ring loss. The findings from this study suggest that it should be a concern for the persistence of fluconazole in the environment.

Introduction

Azole fungicides are a kind of emerging organic pollutants, which have triazoles or imidazoles with benzene ring of different substituents. And they are widely used in personal care products and pharmaceuticals for their high efficacy in antifungal action. Among them, frequently used azole fungicides were imidazole fungicide climbazole and triazole fungicide fluconazole. For example, climbazole is an antifungal

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agent added to some shampoos and body washes, and fluconazole is a medicine used to treat fungal infections in clinical treatment. After use, they are discharged into wastewater treatment plants (WWTPs) along with domestic wastewater. However, conventional WWTPs are designed for removal of common pollutants rather than emerging organic pollutants. Thus, there are limited removals in the conventional WWTPs. The aqueous removal rates of climbazole and fluconazole in WWTPs are about 70% and 10%, respectively (Casado et al., 2014; Chen et al., 2012; Wick et al., 2010). As a result, the WWTPs become a major point source of azole fungicides. It was reported that the usage of climbazole has been estimated to be 345 tons in the whole China, and 245 tons could be discharged into the receiving environment after wastewater treatment (Zhang et al., 2015b). Fluconazole has been estimated to have emission of 17 tons per year in China (Liu et al., 2017). Consequently, they are frequently detected in various environmental media such as surface water and sediment (Iranzo et al., 2018; Liu et al., 2017, 2018). For example, fluconazole and climbazole were detected at 312 ng/L and 656 ng/L in surface water, respectively and at 100 ng/kg and 4000 ng/kg in sediment, respectively (Liu et al., 2017). According to the multimedia fate modeling, approximately 93% of climbazole is discharged into the water compartment and 7% is discharged into the soil compartment (Zhang et al., 2015b). The residual azole fungicides in the receiving aquatic environment may cause unpredicted adverse effects on nontarget organisms for their endocrine disrupting activities (Knebel et al., 2019; Kobayashi et al., 2002; Lv et al., 2017). Therefore, it is essential to understand the removal mechanisms of azole fungicides in WWTPs in order to improve their removal efficiency.

Activated sludge process is the main treatment technology in WWTPs for control of organic pollutants by using anaerobic, anoxic and aerobic treatment processes. Compared to advanced wastewater technologies such as Fenton, ozonation and photocatalysis, activated sludge treatment is economical and easy to be operated. It has been reported that many emerging organic pollutants can be removed by acclimated activated sludge (Min et al., 2018; Wang and Wang, 2018). Therefore, understanding the behavior and fate of azole fungicides in activated sludge process is the key to improve removing efficiency in this process. However, there are few reports of degradation mechanism of azole fungicides in activated sludge process. Though some studies have been conducted on the removal of azole fungicides in WWTPs, they only concentrated on the removal rates in the liquid based on process flow (Chen et al., 2012; Liu et al., 2017). It is still unknown that whether the removal of azole fungicides is due to the adsorption by sludge or degradation by microorganisms. The behavior and fate of azole fungicides in activated sludge need to be thoroughly investigated.

Recently, some computer software and models are available to predict the biotransformation products and biodegradation pathways, which make it easier to understand the biodegradability of target contaminants, and aid in designing biodegradation experiments (Arora and Shi, 2010; Rücker and Kümmerer, 2012). For example, the Eawag-Biocatalysis/Biodegradation Database and Pathway Prediction System (EAWAG-BBD/PPS) and Pathway Prediction Server (Path Pred) can give possible metabolites via general enzyme classes and biodegradation pathways (Latino et al., 2017; Moriya et al., 2010; Wicker et al., 2016). The microbial biodegradation pathways of pesticide carbaryl have been accurately predicted by EAWAG-BBD/PPS (Garg et al., 2014). It even helps to identify degradation bacteria of malathion through pathway prediction system (Sivakumar et al., 2017). Moreover, Metabolite Predict software (Metabolite Tools 2.0, Bruker Daltonics, Bremen, Germany) can also predict metabolites in Phase I, II and Cytochrome P450 reactions (Beretsou et al., 2016; Iatrou et al., 2017).

The aim of this study was to investigate the removal mechanism of two typical azole fungicides with different structures (climbazole and fluconazole) in aerobic activated sludge process with combined experimental and model prediction. The biotransformation products from laboratory experiments were tentatively identified by high resolution mass spectrometry using suspect and non-target screening method. The combination of products identification and pathway prediction tools was applied to interpret the detailed biodegradation pathways. In addition, the toxicity of these biotransformation products was also estimated using the toxicity predictive software of USEPA ECOSAR (v2.0). The results from this study can help better understand biodegradation of azole fungicides in WWTPs.

1. Materials and methods

1.1. Materials and reagents

Climbazole (99.9%) and fluconazole (99.9%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The stock solutions of climbazole and fluconazole were prepared in methanol and stored in −20 °C. All HPLC grade organic solvents, used for sample processing and analysis, were obtained from Merck Corporation (Shanghai, China).

Activated sludge samples were collected from a WWTP in Guangzhou, which processes 28,000 m^3/day wastewater (200,000 population equivalents). The WWTP employs oxidation ditch process, including primary sedimentation basin, oxidation ditch and a final clarifier before discharge of effluent to the nearby river. The mixed liquid suspended solid (MLSS) was 1.6 g/L during the sampling period.

Incubation solutions with 10% of each inoculum (sludge: salt solution, V/V) were prepared in a minimal salts medium consisting of KCl (306 mg/L), $MgCl_2$ (9.5 mg/L), NaCl (35.1 mg/L), $CaCl_2$ (67.7 mg/L), NH_4Cl (900 mg/L), K_2HPO_4 (252 mg/L) and KH_2PO_4 (252 mg/L), amended with trace salts (100 μL/L) and vitamins (1 mL/L) (Liu et al., 2013).

1.2. Biodegradation experiments

The batch experiments were conducted according to the OECD 303A test (Liu et al., 2013). In brief, degradation treatments and controls were set up in 1 L glass flasks containing 500 mL incubation media. The climbazole or fluconazole was spiked into the incubation media with an initial concentration of 500 μg/L. For the chemical control of hydrolysis and volatilization, the target compound was spiked with the same concentration in 500 mL salts solution. For the sterile control of each treatment,

the sludge was autoclaved (120 °C, 20 min) twice followed by addition of the metabolic inhibitor sodium azide (NaN_3, 0.1%, W/V final concentration) (Wang et al., 2017). All the flasks were completely covered by aluminum foil to prevent photodegradation, then incubated at 25 °C in an orbital mixer incubator with continuous shaking at 150 r/min. Triplicate samples (1 mL each) were taken from each bottle at certain time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 days for climbazole and 0,1, 3, 5, 12, 21, 28, 35, 42, 49, 56, 63 and 77 days for fluconazole). All batch experiments were performed in duplicates.

1.3. *Sample extraction*

1.3.1. *Liquid and solid sludge samples*

Climbazole and fluconazole had different pretreatment processes due to their different physiochemical properties. Firstly, 1 mL sample was centrifuged at 10,000 r/min for 3 min (Beckman, USA). For climbazole, the supernatant was transferred into another tube and was extracted using 400 μL ethyl acetate for three times. The extracting solution was dried under gentle nitrogen stream and re-dissolved in mixed solvent of water and methanol (80:20, V/V) before analysis. The remaining solid sludge was extracted twice with 0.5 mL methanol and the extract was dried under a gentle nitrogen stream. It was re-dissolved in mixed solvent of water and methanol (80:20, V/V) for instrumental analysis. The total recovery of climbazole in liquid and sludge was 96.8%. For fluconazole, the supernatant was measured directly after passing through the 0.22 μm water phase filter. The remaining solid sludge was extracted with the same method as climbazole. The concentration of fluconazole in the sludge was below detection limit. The recovery of fluconazole in liquid was 94.8%. All samples were analyzed by an Agilent 1290 Infinity high performance liquid chromatography (HPLC) with a diode array detector. The limit of detection of climbazole and fluconazole by HPLC were both 5 μg/L. The detailed HPLC parameters are shown in Table S1, Supplementary information.

1.3.2. *Sample treatment for products identification*

Every 100 mL reaction solution was sampled and extracted by solid phase extraction (SPE) for biodegradation products analysis. The 100 mL sample was firstly centrifuged at 3000 r/min for 5 min (Beckman, USA). The supernatant was transferred to 300 mL round-bottom flask. And the remaining pellets in the tubes were extracted twice with 10 mL methanol by ultrasonication for 15 min and then extracted one more time with 10 mL methanol/0.1% (V/V) formic acid water solution (5:5, V/V) (Liu et al., 2017). All extracted supernatants were combined and diluted with Milli-Q water (approx. 300 mL) to make the organic solvent content below 10%. The aqueous samples were re-extracted by using Oasis HLB SPE cartridges (200 mg; Waters, Milford, MA). Prior to use, the cartridges were conditioned with 10 mL of methanol and 10 mL of milli-Q water, and aqueous samples were loaded at 1 mL/min. When finished, the SPE cartridges were dried under vacuum for 2 hr, and then eluted with 3 × 2 mL dichloromethane, 3 × 2 mL ethyl acetate, and 3 × 2 mL methanol in sequence. Eluates were dried under a gentle nitrogen stream and re-dissolved in 1 mL methanol before analysis (Liu et al., 2016). The recovery of parent compound climbazole was 82.3%.

1.4. *Identification of transformation products*

Transformation products were identified using suspect and non-target screening method (Beretsou et al., 2016; Iatrou et al., 2017). Suspect database of plausible transformation products was created referring to the published literatures and the prediction results using tools including EAWAG-BBD/PPS, Path Pred and biotransformation mass defects filtering (Agilent Technologies) (Paguigan et al., 2017). All the biodegradation samples including blank and sterile control were analyzed using Agilent 6545 quadrupole time-of-flight mass spectrometer equipped with an Agilent 1290 Infinity HPLC (HPLC-QTOF-MS). The blank sample only has activated sludge and sterile control sample includes sterile activated sludge and spiked target compound. It was operated in the electronic spray ion source (ESI) with positive and negative modes. The details of instrumental conditions are given in Appendix A **Text S1**. Samples were first analyzed using full scan mode and then all the plausible transformation products were further analyzed using targeted MS/MS mode for identification. For data processing, the acquired QTOF-MS data were firstly screened by extracting the exact masses of the potential transformation products according to the established suspect database. Additional transformation products absent in the suspect database were also screened by a non-target approach. Background subtraction and peak picking were carried out using Agilent Mass Hunter Profinder software, which can be used for difference analysis between the blank, control and biodegradation samples with a meaningful time trend. The molecular formula and structures were correlated according to each precise mass fragment ion of MS/MS spectrum using Agilent Mass Hunter molecular structure correlator software. Characteristic fragmentation of each spectrum was used to verify the proposed structures of transformation products. Meanwhile Agilent 7890B-G1033A gas chromatography mass spectrometry (GC–MS) was also used to probe the transformation products (Appendix A **Text S2**), which could be matched by the mass spectrum library provided by National Institute of Standards and Technology. All proposed transformation product structures were assigned a confidence level as it has become convention in the field of non-target analysis (Schymanski et al., 2014).

1.5. *Statistical analysis and model prediction*

The degradation rate was analyzed using one-way analysis of variance (ANOVA) applying the LSD multiple comparison test using IBM SPSS Statistics software (16.0). Physicochemical properties of climbazole and fluconazole were calculated by US EPA EPI Suite™ software (USEPA, 2014). The toxicity of climbazole, fluconazole and their biodegradation products for fish, daphnid and green algae were predicted by using software ECOSAR (v2.0) (Sanderson, 2003). The microbial degradation pathways of climbazole and fluconazole were predicted by EAWAG-BBD/PPS system (Wicker et al., 2016).

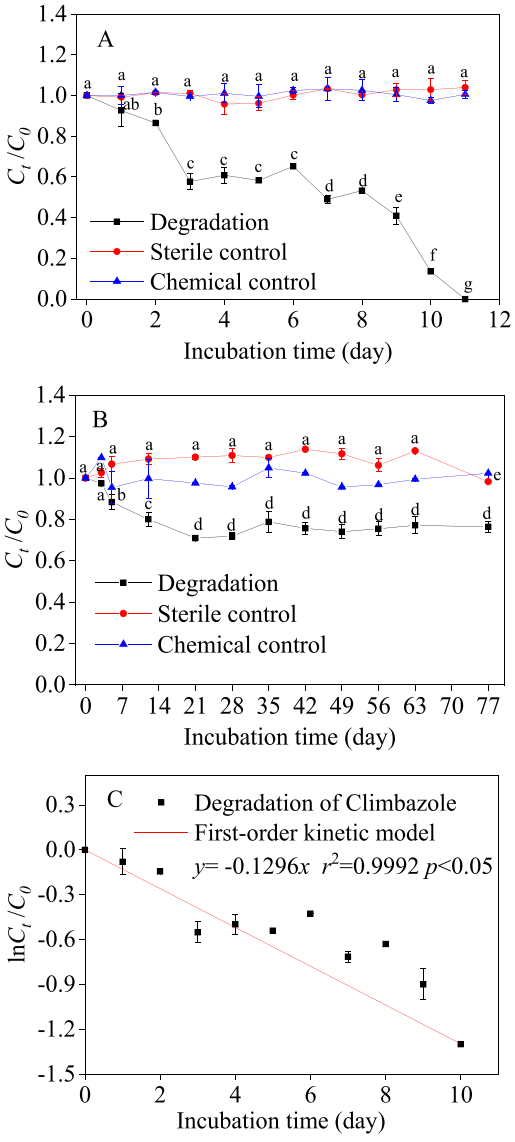

Fig. 1 – Biodegradation of climbazole (A) and fluconazole (B), and the first-order kinetic model of climbazole (C) in the activated sludge system under aerobic conditions. Experiment conditions: $[chemicals]_0$ = 500 μg/L, MLSS=160 mg/L, V = 500 mL, 25 °C in an orbital mixer incubator with continuous shaking at 150 r/min. Error bars represent standard error of mean. Data with different letters indicate significant difference (p<0.05) with reaction time.

2. Results and discussion

2.1. *Degradation of climbazole and fluconazole*

The degradation of climbazole and fluconazole in the aerobic activated sludge system is shown in Fig. 1. There was no significant difference between the controls. Statistical labels on data points for time effect in chemical control were not shown in figures. There are two ways of abiotic and biological processes to remove pollutants in activated sludge (Wei et al., 2019). Abiotic process includes hydrolysis, photolysis, evaporation, and sludge adsorption. Both climbazole and fluconazole were found stable in chemical control group as shown in Fig. 1**A** and Fig. 1**B**. This suggested that there were no hydrolysis and evaporation for both climbazole and fluconazole, and no photolysis as well since the system was covered by aluminum foil. Climbazole and fluconazole were also found to be persistent in the sterile control group, indicating that no degradation of climbazole and fluconazole by chemical processes occurred in the sterile sludge. Therefore, only biological process could be responsible for any loss in nonsterile sludge treatments. Climbazole was quickly degraded in the first 3 days and completely degraded at the 11th day. The degradation of climbazole in the aerobic activated sludge system was fit to the first-order kinetic model as shown in Fig. 1**C**. The half-life of climbazole degradation was calculated to be 5.3 days. However, only 30% of fluconazole was found degraded in first 3 weeks and then kept unchanged for another 8 weeks. But the hydraulic retention time of WWTPs is usually not so long. Thus, the persistence of fluconazole in aerobic active sludge from the present study was consistent with a previous report (Kahle et al., 2008).

The concentration changes of climbazole in the liquid and solid sludge during the biodegradation are shown in Fig. 2. In the sterile group (Fig. 2**A**), the concentration of climbazole in the liquid phase decreased in the first two days, while the concentration of climbazole in the sludge phase increased due to its sorption from liquid. The concentrations of climbazole in both phases remained dynamically stable in the following time. And the total concentration of climbazole in the sterile group was similar to the spiked value. In the degradation group (Fig. 2**B**), the concentration of climbazole in the liquid decreased quickly with the incubation time, while the concentration of climbazole in the solid also increased in the first two days due to the sorption from liquid and then decreased quickly.

Appendix A **Fig. S1** shows the concentration changes of fluconazole in the liquid during the biodegradation. The concentration of fluconazole in the liquid of sterile group was similar to the spiked concentration, without any detection in the solid samples. The concentration of fluconazole in the degradation group decreased in first 3 weeks and then remained unchangeable in the following time.

The different degradation behaviors for climbazole and fluconazole in activated sludge system depend on their physiochemical properties and chemical structures (Sabljić et al., 1995). Climbazole could adsorb to the sludge due to its high octanol-water partition coefficient with $\log K_{ow}$ of 3.8 (Appendix A **Table S2**). While the fluconazole tends to stay in the liquid because of its low octanol-water partition coefficient ($\log K_{ow}$ 0.5, Appendix A **Table S2**). This is consistent with a previous sorption study of fluconazole (Fountouli and Chrysikopoulos, 2018). In terms of chemical structure, climbazole is an imidazole fungicide, while fluconazole is triazole fungicide. For imidazole fungicides, it is easy to have azole ring loss and ring cleavage. For triazole fungicides, there is no change of azole ring and hydroxylation only occurred at other active moiety of compounds (Rosch et al., 2016). So for fluconazole, reaction only occurred at benzene ring. But the presence of electron-withdrawing group (like

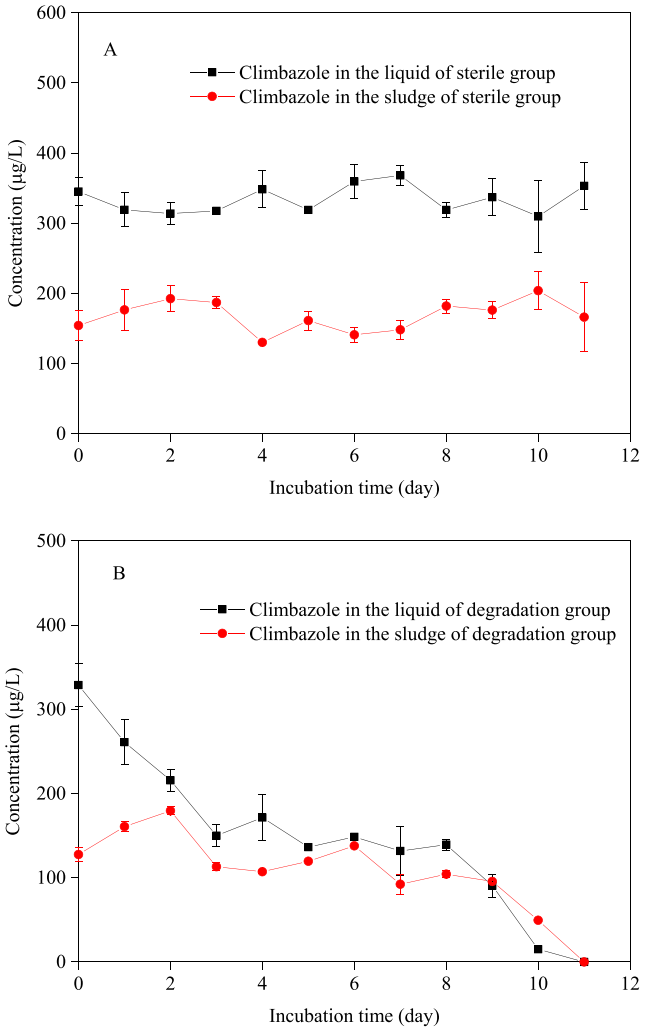

Fig. 2 – Concentrations of climbazole in the liquid and solid sludge of sterile group (A) and degradation group (B). Experiment conditions: [chemicals]$_0$ = 500 μg/L, MLSS=160 mg/L, V = 500 mL, 25 °C in an orbital mixer incubator with continuous shaking at 150 r/min. Error bars represent standard error of mean.

halogens [-F, -Cl] and nitrogen) can deactivate the aromatic ring in certain positions for attack by oxygenase, thus resulting in lower biodegradation rate (Acharya et al., 2019). Fluorine atom in fluconazole is more electronegative than chlorine atom in climbazole. The fluorine withdraws electrons from the aromatic ring, which makes fluconazole more difficult to react with peroxidases and resulting in lower oxidation ability (Suzuki et al., 2001). Same as the present study, no biotransformation products of fluconazole were reported in a previous biodegradation study of azole fungicides in aquatic invertebrate gammarus pulex (Rosch et al., 2016). Another chemical flutriafol with a similar chemical structure to fluconazole was reported with a very long degradation half-life in soil and sediment (White et al., 2010; Zhang et al., 2015a). Therefore, more attention should be paid to fluconazole that is resistant to microbial degradation.

2.2. *Identification of transformation products*

Transformation products and pathways of climbazole and fluconazole were firstly predicted by the EAWAG-BBD/PPS, as shown in Appendix A **Figs. S2** and **S3**. There are different transformation routes with 25 and 11 of products for climbazole and fluconazole, respectively. Based on the prediction, climbazole is more likely to be biodegraded than fluconazole by microbes. However, only ten biodegradation products of climbazole were identified from the laboratory experiments using suspect and non-target screening method. Among them, nine transformation products were identified by HPLC-QTOF-MS and one was identified by GC–MS. Biodegradation products of fluconazole were not identified in aerobic activated sludge.

Based on the accurate masses of protonated molecules and MS/MS fragments, the empirical formulas, chemical structures of transformation products for climbazole were tentatively proposed, as shown in Table 1. The detailed mass spectra of ten transformation products are shown in Appendix A **Figs. S4-S5**. The evolution profiles of these products following different incubation time are shown in Appendix A **Fig. S6**. The abundance of the transformation products increased with the incubation time except TP111, TP259 and TP275. The abundance of these three products increased in first 4 days and then decreased due to further degradation.

The structure analysis of the ten transformation products is presented below. TP69, TP111, TP253, TP267 and TP295 were first reported as transformation products of climbazole in this study. TP253, TP267 and TP295 were not present in the suspect database. They were identified by the non-target approach. For TP253, there is a characteristic fragment with mass-to-charge ratio (*m/z*) of 126.0420 amu ($C_5H_5N_2O_2^+$) (Appendix A **Fig. S4D**), which can be formed by cleavage of the parachlorophenol (C_6H_4ClO). TP267 has a characteristic fragment with *m/z* of 199.0157 amu ($C_9H_7ClO_3^+$), which is due to the loss of the imidazole ($C_3N_2H_3$) (Appendix A **Fig. S4F**). TP295 can also lose the imidazole to form the characteristic fragment with *m/z* of 227.0834 amu ($C_{12}H_{15}ClO_2^+$) (Appendix A **Fig. S4H**).

Products TP69, TP111 and TP128 were identified with the help of pathway prediction tool of EAWAG-BBD/PPS. They were present in the suspect database. TP69 and TP111 were screened by extracting the exact masses and confirmed in the targeted MS/MS mode of HPLC-QTOF-MS. TP128 was identified using the GC–MS, which was matched with the spectrum of 4-chlorophenol in the NIST library (Appendix A **Fig. S5**). It has been reported in the study of UV photolysis of climbazole by Castro et al. (2016), which was further confirmed using the standard chemical. TP183, TP259, TP275 and TP309 had been reported in the degradation of climbazole by the advanced oxidation process (Cai et al., 2019; Castro et al., 2016; Liu et al., 2016). For TP183, there is a characteristic fragment with *m/z* of 126.0917 amu ($C_5H_5N_2O_2^+$) (Appendix A **Fig. S4C**), which is produced by cleavage the tertiary butyl ($C(CH_3)_3$). TP259 has characteristic fragments with *m/z* of 191.1057 amu ($C_{12}H_{15}O_2^+$) and 163.1120 amu ($C_{11}H_{14}O^+$) (Appendix A **Fig. S4E**). Fragment 191.1057 amu is generated by breakage of imidazole in TP259. Fragment 163.1120 amu is formed by decarbonylation and methyl of 191.1057 amu. The MS/MS spectrum of TP259 is the same as that found in the report of

Table 1 – Biodegradation products of climbazole in the activated sludge under aerobic condition.

Compounds	Retention time (min)	Experimental *m/z*	Proposed formula	Error (ppm)	DBE	MS/MS Fragments	Confidence level*	References
climbazole	16.66	293.1054	$C_{15}H_{17}ClN_2O_2$	−1.3	8	197.0727 166.1100	1	Castro et al., 2016
TP69	14.40	69.0451	$C_3H_4N_2$	0.1	3	69.0451	2b	This study
TP111	12.05	111.0441	$C_6H_6O_2$	0.5	4	55.0542 71.9295	2a	This study
TP183	3.25	183.1129	$C_9H_{14}N_2O_2$	0.2	4	126.0917 101.95	2a	Liu et al., 2016
TP253	6.73	253.0375	$C_{11}H_9ClN_2O_3$	0.6	8	126.0420 166.9893	2b	This study
TP259	13.87	259.1445	$C_{15}H_{18}N_2O_2$	−5.0	8	163.1120 191.1057	2a	Cai et al., 2019; Castro et al., 2016; Liu et al., 2016
TP267	10.70	267.0532	$C_{12}H_{11}ClN_2O_3$	−0.4	8	166.9893 199.0157	2b	This study
TP275	11.37	275.1386	$C_{15}H_{19}N_2O_3$	−1.8	8	179.1060 109.0284	2a	Cai et al., 2019; Castro et al., 2016; Liu et al., 2016
TP295	14.63	295.1190	$C_{15}H_{19}ClN_2O_2$	6.0	7	264.0766 99.0805	2b	This study
TP309	17.16	309.0662	$C_{15}H_{17}ClN_2O_3$	−0.5	8	225.0684 197.0728	3	Cai et al., 2019
TP128	9.44	128.0	C_6H_5ClO	0.1	4	GC-EI-MS	2a	2016

BDE: Double Bond Equivalent.
* Reference Schymanski et al., 2014.

Castro et al. (2016). The characteristic fragment of TP275 (Appendix A **Fig. S4G**) is also the same as our previous report of Cai et al. (2019). TP309 has a characteristic fragment with *m/z* of 225.0684 amu ($C_{12}H_{13}ClO_2^+$).

According to the guidelines of non-target analysis (Schymanski et al., 2014), TP69, TP253, TP267 and TP295 were newly identified as the transformation products of climbazole, with no standards or literature information available for confirmation. Thus, they were assigned with an identification level 2b. TP111, TP128, TP183, TP259 and TP275 could match spectrum data in literature or library, which were assigned with an identification level 2a. TP309 had insufficient information for one exact structure because of uncertain position of hydroxyl, which was assigned with an identification level 3.

2.3. *Proposed biotransformation pathways*

Based on the products identification and pathway prediction, the possible biodegradation pathways for climbazole were proposed, as shown in Fig. 3. The biotransformation of climbazole can be divided into route A and route B. In route A, hydroxylation of climbazole can lead to the formation of TP309. The cleavage of C–O bond of climbazole leads to generation of TP183 and TP128. TP183 can be further oxidized and degraded into a small molecule TP69 and other non-identified products. The chlorine of TP128 is replaced by the hydroxyl group to form TP111. Ring cleavage of climbazole can directly produce TP69 and intermediate product IM240 (undetected). Oxidative dechlorination of climbazole can produce TP259. TP275 could be generated from hydroxylation of TP259. But the abundance of TP 275 was very low. It was predicted that it could be further degraded into TP 69, TP183 and TP111 due to the similar structure as climbazole. In route B, TP253, TP267 and TP295 can be formed from oxidizing side chain alkyl of climbazole.

Transformation pathway is usually proposed based on semi-quantitative and structural changes (Beretsou et al., 2016), which does not provide detailed explanations. The proposed biotransformation pathways of climbazole were explained reasonably by the predicted pathways of EAWAG-BBD/PPS in this study. EAWAG-BBD/PPS can be used for predicting plausible pathways of chemicals by microbial degradation, which is based on the biotransformation rules. All the rules are based on the reactions found in the EAWAG-BBD database or in the scientific literature. An accurate prediction depends on compounds with similar structures whose biodegradation pathways are reported in the scientific literature. Appendix A **Table S3** shows the biotransformation reaction rules in the proposed pathway of climbazole. Detailed

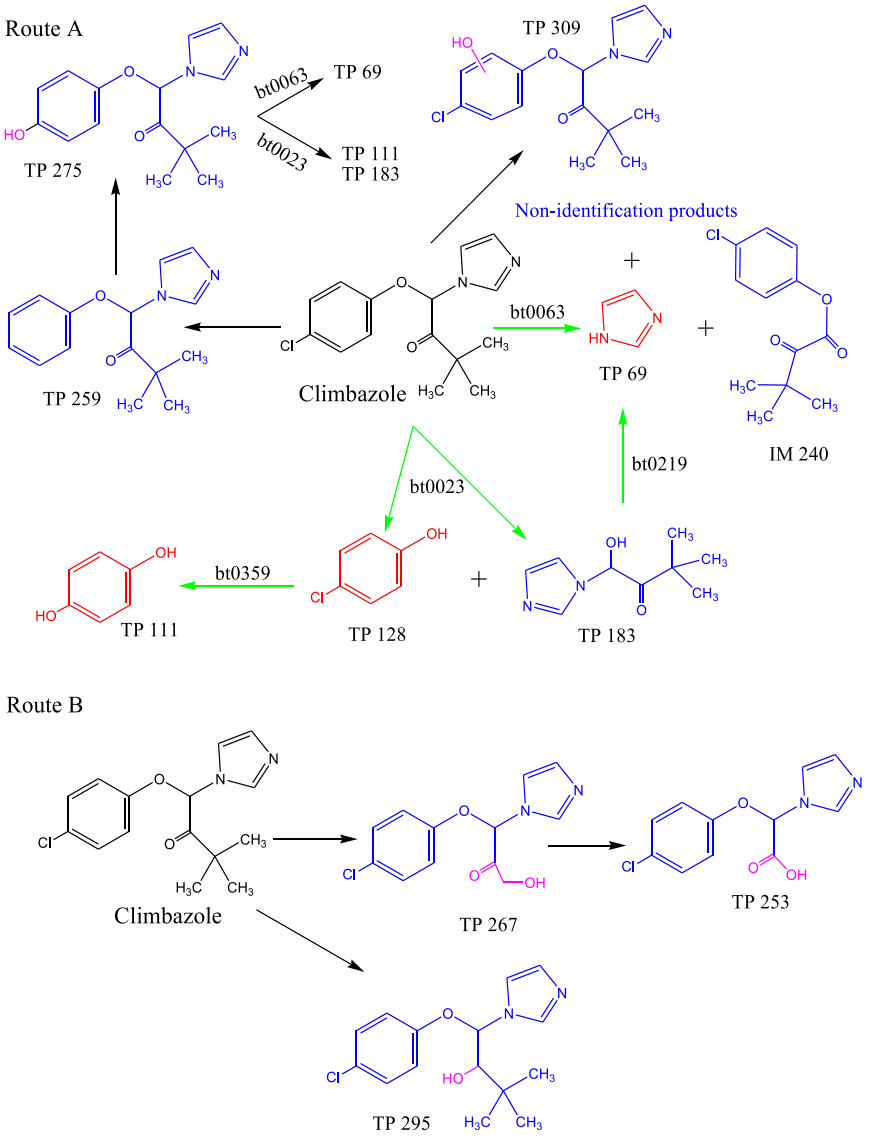

Fig. 3 – Proposed biodegradation pathways of climbazole in the activated sludge. Green arrows indicate that they are consistent with the prediction pathways. Red molecules are consistent with the prediction results. Number with bt are biotransformation rules.IM: intermediate product.

explanation of the rules follows below. TP128 and TP183 can be formed by biodegradation of climbazole with the biotransformation rule bt0023. For a chemical of aromatic-aliphatic ether, it can be biodegraded into phenol derivative (TP128) and aldehyde. TP183 has the hydroxyl while the prediction structure is aldehyde group. TP183 with the structure of hemiaminal can be degraded into TP69 and other non-identified products with the biotransformation rule bt0219 (Cleavage of hemiaminal to amine and aldehyde). Parachlorphenol TP128 can be transformed to hydroquinone TP111 with the biotransformation rule bt0359 (Oxidation of 4-Halophenol to hydroquinone). Biodegradation of climbazole can directly produce TP69 with IM240 (intermediate product) by biotransformation rule bt0063. For a chemical with structure of secondary amine, it could be degraded via cleavage of amine and aldehyde or ketone. Ring cleavages or ring losses are always observed in chemicals with imidazole which have two nitrogen atoms (Rosch et al., 2016). However, IM240 was not be found in this experiment, possibly due to its unstable structure of lactone (4-chlorophenoxy-butanoic acid), which is easily biodegradation by microbes (Safari et al., 2014). Moreover, TP309 was generated from hydroxylation of climbazole. Hydroxylation is the most prevalent reaction observed for all azoles and occurs at the aliphatic part of the molecule, the chlorophenyl moiety and the azole ring (Choi and Oh, 2019; Pimviriyakul et al., 2020). In some cases, hydroxylation products can be further oxidized to form ketones (Rosch et al., 2016). Biodegradation of climbazole by microbes were consistent with biotransformation study of azole in aquatic invertebrate gammarus pulex, the main biotransformation routes of imdazoles were azole ring loss and hydroxylation occurring at side chain (Rosch et al., 2016).

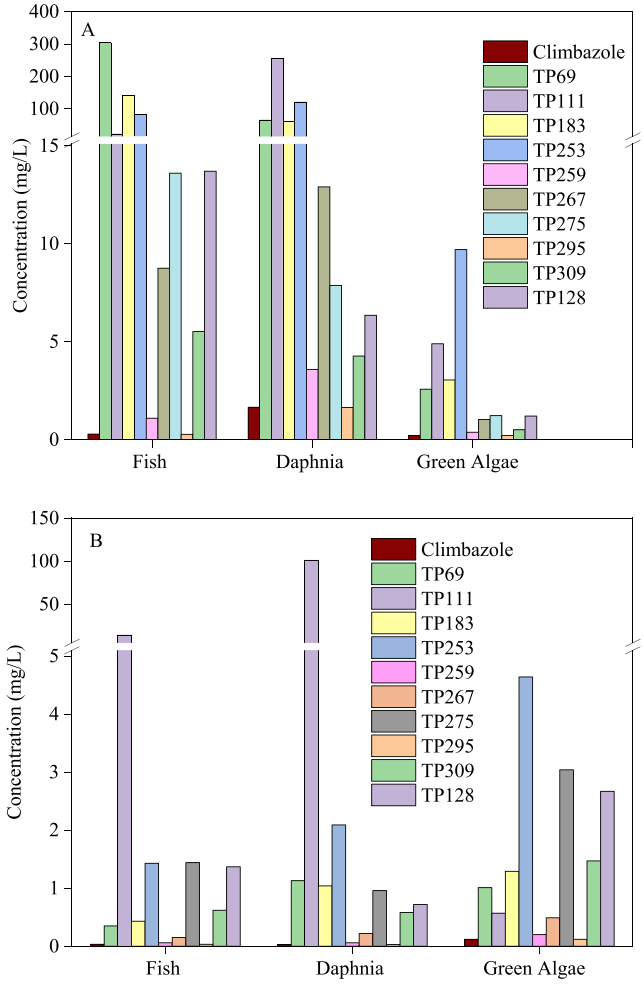

Fig. 4 – Predicted EC_{50} values (A) and chronic toxicity values (ChVs) (B) of climbazole and its transformation products for aquatic organism obtained through USEPA ECOSAR.

2.4. *Toxicity assessment*

The acute and chronic toxicity of climbazole and their biotransformation products to aquatic organisms including fish, daphnia and green algae were predicted by USEPA ECOSAR (v2.0). The corresponding toxic values of climbazole are shown in Fig. 4. The acute toxicity of climbazole to aquatic organisms was evaluated with the median effective concentrations (EC_{50}), as shown in Fig. 4**A**. The EC_{50} of climbazole to fish (96 hr), daphnia (48 hr) and green algae (96 hr) was 0.29 mg/L, 1.66 mg/L and 0.22 mg/L, respectively. The EC_{50} values of transformation products were 5–1000 times higher than that of climbazole except TP295 whose structure is similar to the climbazole. Thus, the acute toxicity of transformation products decreased substantially than climbazole itself. The chronic toxicity values (ChVs) of climbazole to aquatic organisms are shown in Fig. 4**B**. Similarly, the ChVs of transformation products to aquatic organisms were 3–3000 times of climbazole, indicating the decreased chronic toxicity of the transformation products.

The acute and chronic toxicity values of fluconazole to aquatic organisms including fish, daphnia and green algae were also predicted. The EC50 of fluconazole to fish (96 hr), daphnia (48 hr) and green algae (96 hr) were 1630 mg/L, 530 mg/L and 55.4 mg/L, respectively. While the ChVs of fluconazole to fish (96 hr), daphnia (48 hr) and green algae (96 hr) were 2.65 mg/L, 66.3 mg/L and 9.93 mg/L, respectively. From the EC50 and ChVs, the toxicity of fluconazole was much smaller than climbazole. But it is persistent in WWTPs, with poor biodegradability. Residual fluconazole might pose potential risks to organisms in the environment. It has been testified that fluconazole had teratogenic effects on rat embryos at very low concentrations (Di Renzo et al., 2007). And it could inhibit gene expression in mouse embryos (Tiboni et al., 2009). Climbazole could be biodegraded and the toxicity of transformation products could be reduced. Compared with the risks of climbazole, more attention should be paid to the persistent fluconazole and related chemicals with similar structure in the environment. It is essential to find a practical way to remove these compounds and reduce adverse effects in the receiving environment. Therefore, more future research is needed to find other degradation ways for fluconazole and related chemicals.

3. Conclusions

The results from this study showed that imidazole fungicide climbazole could be degraded while triazole fungicide fluconazole was persistent in the aerobic activated sludge. The different biodegradation behaviors between climbazole and fluconazole in the activated sludge could be explained by their physicochemical properties and molecular structures. For climbazole, the main biotransformation routes were azole ring loss and hydroxylation occurring at active moiety. For fluconazole, it was found difficult to be biodegraded due to the presence of two triazole rings and fluorine substituted groups in the benzene ring. Although these two chemicals belong the same group of azole fungicides, they could have complete different environmental behaviors and biological effects in the environment. This provides a scientific basis for relevant agencies to take different management approaches.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.11.007.

REFERENCES

Acharya, K., Werner, D., Dolfing, J., Barycki, M., Meynet, P., Mrozik, W., et al., 2019. A quantitative structure-biodegradation relationship (QSBR) approach to predict biodegradation rates of aromatic chemicals. Water Res. 157, 181–190.

Arora, P.K., Shi, W.X., 2010. Tools of bioinformatics in biodegradation. Rev. Environ. Sci. BioTechnol. 9, 211–213.

Beretsou, V.G., Psoma, A.K., Gago-Ferrero, P., Aalizadeh, R., Fenner, K., Thomaidis, N.S., 2016. Identification of biotransformation products of citalopram formed in activated sludge. Water Res. 103, 205–214.

Cai, W.W., Peng, T., Zhang, J.N., Hu, L.X., Yang, B., Yang, Y.Y., et al., 2019. Degradation of climbazole by UV/chlorine process: kinetics, transformation pathway and toxicity evaluation. Chemosphere 219, 243–249.

Casado, J., Rodriguez, I., Ramil, M., Cela, R., 2014. Selective determination of antimycotic drugs in environmental water samples by mixed-mode solid-phase extraction and liquid chromatography quadrupole time-of-flight mass spectrometry. J. Chromatogr. A 1339, 42–49.

Castro, G., Casado, J., Rodriguez, I., Ramil, M., Ferradas, A., Cela, R., 2016. Time-of-flight mass spectrometry assessment of fluconazole and climbazole UV and UV/H_2O_2 degradability: kinetics study and transformation products elucidation. Water Res. 88, 681–690.

Chen, Z.F., Ying, G.G., Lai, H.J., Chen, F., Su, H.C., Liu, Y.S., et al., 2012. Determination of biocides in different environmental matrices by use of ultra-high-performance liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 404, 3175–3188.

Choi, D., Oh, S., 2019. Removal of chloroxylenol disinfectant by an activated sludge microbial community. Microbes Environ. 34, 129–135.

Di Renzo, F., Broccia, M.L., Giavini, E., Menegola, E., 2007. Citral, an inhibitor of retinoic acid synthesis, attenuates the frequency and severity of branchial arch abnormalities induced by triazole-derivative fluconazole in rat embryos cultured in vitro. Reprod. Toxicol. 24, 326–332.

Fountouli, T.V., Chrysikopoulos, C.V., 2018. Adsorption and thermodynamics of pharmaceuticals, acyclovir and fluconazole, onto quartz sand under static and dynamic conditions. Environ. Eng. Sci. 35, 909–917.

Garg, V., Khan, S., Dutt, K., 2014. Systematic analysis of microbial degradation pathway of 1-naphthyl-n-methyl carbamate generated by EAWAG biocatalysis/biodegradation database-pathway prediction system. Int. J. Appl. Sci. Res. Rev. 1, 049–055.

Iatrou, E.I., Gatidou, G., Damalas, D., Thomaidis, N.S., Stasinakis, A.S., 2017. Fate of antimicrobials in duckweed Lemna minor wastewater treatment systems. J. Hazard. Mater. 330, 116–126.

Iranzo, M., Gamon, M., Boluda, R., Mormeneo, S., 2018. Analysis of pharmaceutical biodegradation of WWTP sludge using composting and identification of certain microorganisms involved in the process. Sci. Total Environ. 640-641, 840–848.

Kahle, M., Buerge, I.J., Hauser, A., Müller, M.D., Poiger, T., 2008. Azole fungicides: occurrence and fate in wastewater and surface waters. Environ. Sci. Technol. 42, 7193–7200.

Knebel, C., Heise, T., Zanger, U.M., Lampen, A., Marx-Stoelting, P., Braeuning, A., 2019. The azole fungicide tebuconazole affects human CYP1A1 and CYP1A2 expression by an aryl hydrocarbon receptor-dependent pathway. Food Chem. Toxicol. 123, 481–491.

Kobayashi, D., Kondo, K., Uehara, N., Otokozawa, S., Tsuji, N., Yagihashi, A., et al., 2002. Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. Antimicrob. Agents Chemother. 46, 3113–3117.

Latino, D.A., Wicker, J., Gutlein, M., Schmid, E., Kramer, S., Fenner, K., 2017. Eawag-soil in enviPath: a new resource for exploring regulatory pesticide soil biodegradation pathways and half-life data. Environ. Sci. Process Impacts 19, 449–464.

Liu, W.R., Ying, G.G., Zhao, J.L., Liu, Y.S., Hu, L.X., Yao, L., et al., 2016. Photodegradation of the azole fungicide climbazole by ultraviolet irradiation under different conditions: kinetics, mechanism and toxicity evaluation. J. Hazard. Mater. 318, 794–801.

Liu, W.R., Yang, Y.Y., Liu, Y.S., Zhang, L.J., Zhao, J.L., Zhang, Q.Q., et al., 2017. Biocides in wastewater treatment plants: mass balance analysis and pollution load estimation. J. Hazard. Mater. 329, 310–320.

Liu, W.R., Yang, Y.Y., Liu, Y.S., Zhao, J.L., Zhang, Q.Q., Yao, L., et al., 2018. Biocides in the river system of a highly urbanized region: a systematic investigation involving runoff input. Sci. Total Environ. 624, 1023–1030.

Liu, Y.S., Ying, G.G., Shareef, A., Kookana, R.S., 2013. Biodegradation of three selected benzotriazoles in aquifer materials under aerobic and anaerobic conditions. J. Contam. Hydrol. 151, 131–139.

Lv, X., Pan, L.M., Wang, J.Y., Lu, L.P., Yan, W.L., Zhu, Y.Y., et al., 2017. Effects of triazole fungicides on androgenic disruption and CYP3A4 enzyme activity. Environ. Pollut. 222, 504–512.

Min, X.B., Li, W., Wei, Z.S., Spinney, R., Dionysiou, D.D., Seo, Y., et al., 2018. Sorption and biodegradation of pharmaceuticals in aerobic activated sludge system: a combined experimental and theoretical mechanistic study. Chem. Eng. J. 342, 211–219.

Moriya, Y., Shigemizu, D., Hattori, M., Tokimatsu, T., Kotera, M., Goto, S., et al., 2010. PathPred: an enzyme-catalyzed metabolic pathway prediction server. Nucl. Acids Res. 38, 138–143.

Paguigan, N.D., El-Elimat, T., Kao, D., Raja, H.A., Pearce, C.J., Oberlies, N.H., 2017. Enhanced dereplication of fungal cultures via use of mass defect filtering. J. Antibiot. (Tokyo) 70, 553–561.

Pimviriyakul, P., Wongnate, T., Tinikul, R., Chaiyen, P., 2020. Microbial degradation of halogenated aromatics: molecular mechanisms and enzymatic reactions. Microb. Biotechnol. 13, 67–86.

Rosch, A., Anliker, S., Hollender, J., 2016. How biotransformation influences toxicokinetics of azole fungicides in the aquatic invertebrate gammarus pulex. Environ. Sci. Technol. 50, 7175–7188.

Rücker, C., Kümmerer, K., 2012. Modeling and predicting aquatic aerobic biodegradation – a review from a user's perspective. Green Chem. 14, 875–887.

Sabljić, A., Güsten, H., Verhaar, H., Hermens, J., 1995. QSAR modelling of soil sorption. Improvements and systematics of log Koc vs. log Kow correlations. Chemosphere 31, 4489–4514.

Safari, M., Amache, R., Esmaeilishirazifard, E., Keshavarz, T., 2014. Microbial metabolism of quorum-sensing molecules acyl-homoserine lactones, γ-heptalactone and other lactones. Appl. Microbiol. Biotechnol. 98, 3401–3412.

Sanderson, H., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicol. Lett. 144, 383–395.

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., et al., 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. Environ. Sci. Technol. 48, 2097–2098.

Sivakumar, S., Anitha, P., Ramesh, B., Suresh, G., 2017. Analysis of EAWAG-BBD pathway prediction system for the identification of malathion degrading microbes. Bioinformation 13, 73–77.

Suzuki, T., Timofei, S., Kurunczi, L., Dietze, U., Schüürmann, G., 2001. Correlation of aerobic biodegradability of sulfonated azo dyes with the chemical structure. Chemosphere 45, 1–9.

Tiboni, G.M., Marotta, F., Carletti, E., 2009. Fluconazole alters CYP26 gene expression in mouse embryos. Reprod. Toxicol. 27, 199–202.
Wang, L., Qiang, Z.M., Li, Y.A., Ben, W.W., 2017. An insight into the removal of fluoroquinolones in activated sludge process: sorption and biodegradation characteristics. J. Environ. Sci. (China) 56, 263–271.
Wang, S.Z., Wang, J.L., 2018. Degradation of emerging contaminants by acclimated activated sludge. Environ. Technol. 39 (15), 1985–1993.
Wei, Z.S., Li, W., Zhao, D.Y., Seo, Y., Spinney, R., Dionysiou, D.D., et al., 2019. Electrophilicity index as a critical indicator for the biodegradation of the pharmaceuticals in aerobic activated sludge processes. Water Res. 160, 10–17.
White, P.M., Potter, T.L., Culbreath, A.K., 2010. Fungicide dissipation and impact on metolachlor aerobic soil degradation and soil microbial dynamics. Sci. Total Environ. 408, 1393–1402.
Wick, A., Fink, G., Ternes, T.A., 2010. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1217, 2088–2103.
Wicker, J., Lorsbach, T., Gutlein, M., Schmid, E., Latino, D., Kramer, S., et al., 2016. enviPath–the environmental contaminant biotransformation pathway resource. Nucl. Acids Res. 44, D502–D508.
Zhang, Q., Zhou, L.L., Yang, Y., Hua, X.D., Shi, H.Y., Wang, M.H., 2015a. Study on the stereoselective degradation of three triazole fungicides in sediment. Ecotoxicol. Environ. Saf. 117, 1–6.
Zhang, Q.Q., Ying, G.G., Chen, Z.F., Liu, Y.S., Liu, W.R., Zhao, J.L., 2015b. Multimedia fate modeling and risk assessment of a commonly used azole fungicide climbazole at the river basin scale in China. Sci. Total Environ. 520, 39–48.