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Degradation of the antifungal pharmaceutical clotrimazole by UVC and vacuum-UV irradiation: Kinetics, transformation products and attenuation of toxicity

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ABSTRACT

Azole fungicides are frequently detected as water contaminants due to extensive societal use, and these broad-spectrum antifungal chemicals can cause harmful effects in non-target organisms. Vacuum ultraviolet (VUV) treatment is an efficient and green technology that produces hydroxyl radical to remove contaminants from water. This study investigated the potential of UVC (254 nm) and combined VUV/UVC (185/254 nm) treatment to remove the widely used antifungal compound clotrimazole from water. Degradation kinetics, degradation mechanisms, and toxicity mitigation were investigated using a VUV photoreactor. Toxicity of clotrimazole to aquatic organisms before and after UV treatment were investigated using the luminescent bacterium *Aliivibrio fischeri*, the bioluminescent yeast *Saccharomyces cerevisiae* BLYR, the filamentous fungus *Fusarium graminearum*, the freshwater microalga *Raphidocelis subcapitata*, and the crustacean *Daphnia magna*. VUV irradiation efficiently degraded the persistent pollutant clotrimazole at elevated concentrations (mg/L) and at environmental concentrations (μg/L) with > 50% abatement in 1 min and > 95% removal within 32 min. VUV photolysis produced 8 transformation products manly resulting from drug hydroxylation in the phenyl ring and/or imidazole group followed by ring opening or loss of the imidazole moiety. Substantial decrease in aquatic toxicity was observed after UV treatment suggesting that VUV irradiation of aqueous clotrimazole generated less-toxic transformation products.

1. Introduction

Azole fungicides are active ingredients used in pharmaceutical and personal care products for the treatment of human mycosis and are also used in agriculture against fungal infections. As a consequence of the extensive use over the past years, these compounds have emerged as a new class of environmental pollutants [1,2]. Mainly released via urban and hospital wastewater, surface runoff and pesticide application, significant amounts of azoles have been emitted to aquatic environments. Azoles fungicides are usually moderately lipophilic and relatively persistent with typical half-lives of weeks to months, and are only partially removed in traditional wastewater treatment plants (WWTPs) [3]. Consequently, WWTPs act as a constant source of azoles into the natural environment where they may have adverse effects on non-target organisms.

Azoles molecules contain a five members cycle and can be classified into triazoles (three nitrogen atoms) or imidazoles (two nitrogen atoms). Clotrimazole is an imidazole antifungal agent that is widely used, to treat different types of skin fungus infections and oral/vaginal candidiasis. Result of the topical application in the form of a cream is easily released into urban wastewater after use in personal hygiene [4]. For example, Roberts et al. [5] investigated the presence of pharmaceuticals in the Tyne River (UK) and reported the presence of clotrimazole in all samples with an average concentration of 21 ng/L. Despite a high frequency of detection in aqueous environment and a high degree of persistence with half-lifes of more than 60 days [6,7], questions remains about the potential harmful impacts [3,8,9]. Although dilution and biotransformation processes will decrease environmental concentrations, azoles have been frequently reported in water as well as in biota [2,10]. For instance, three azole fungicides including climbazole,

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carbendazim and clotrimazole have been detected in fish muscle tissues [11,12]. Moreover, azole exposure has been related to the reduction of fish growth [13], and to the masculinization of wild fish populations [14]. Azole fungicides may also pose a risk to other non-target organisms including aquatic macrophytes and algae [1,7,15,16]. Furthermore, there is also evidence of azoles involvement in development of antifungal resistance with potential impact on human health [17,18].

Many technologies can be applied to remove micropollutants from the urban water cycle including advanced oxidation processes (AOP), such as photocatalytic processes [19–21], Fenton-like processes [22] and electrocatalysis [23], among others. Ultraviolet (UV) based AOPs have been used for water disinfection and has appeared as a promising technology to remove unwanted chemicals from the water cycle and can potentially mitigate environmental impacts [24]. Many UV based AOPs require the addition of chemical agents (e.g., supplementary oxidants and catalysts) for the generation of reactive species able to react with chemical pollutants. The need for adding chemical reactants has some potential drawbacks in large scale operations including cost effectiveness, practical aspects of regeneration and occasional toxicity of the treated water. Vacuum-UV (VUV) technology is based on UV radiation at wavelength lower than 200 nm that allow the in-situ formation of reactive species, manly hydroxyl radicals ($^{\bullet}OH$) by direct water dissociation without chemical addition [24,25]. Common VUV lamps are characterized by the emission at 185 nm (VUV) and radiation at 254 nm (UVC), and represent a promising technology for organic micropollutant removal in treatment of, e.g., wastewater and drinking water [26–29].

Despite the efficiency of different AOPs in degrading chemical pollutants, concerns are raised about the persistence and ecotoxicity of their photoproducts. As an example, Boxall et al. [30] analysing the toxicity of hundreds of photoproducts of several synthetic chemicals, observed that even if the majority of the products showed comparable or lower toxicity compared with the parent molecule, 20% were $>$ 3 times more toxic while 9% were $>$ 10 times more toxic than the parent molecule. Therefore, it is of high importance to investigate the potential toxicity of the resulting photoproducts in AOP processes. To the best of our knowledge, there is no current information about clotrimazole removal under VUV irradiation and the generation of photoproducts and associated changes in ecotoxicity. Due to the widespread use in society and environmental occurrence, it appears relevant to assess the efficiency of VUV technologies to remediate aqueous antifungal clotrimazole and to mitigate the toxicant property including the drug intermediates.

In this study, we examined the effect of UVC and VUV irradiation of clotrimazole in water on degradation, occurrence of transformation products and changes in ecotoxicity. A specific aim was to determine to what degree UV irradiation could facilitate direct or indirect photolysis and thereby attenuate toxicity of clotrimazole to different aquatic test organisms. Changes in toxic responses were compared before and after UVC and VUV irradiation using a battery of organisms that included *Aliivibrio fischeri*, *Raphidocelis subcapitata, Saccharomyces cerevisiae, Fusarium graminearum* and *Daphnia magna.* Organisms from different trophic levels were included to better assess biological effects of all bioactive compounds in samples with clotrimazole after UV treatment including transformation products. To the best of our knowledge, this is the first study comparing UV mediated photolysis and changes in ecotoxicity of clotrimazole after VUV/UVC treatment.

2. Materials and methods

2.1. Chemicals

Clotrimazole [1-[(2-Chlorophenyl)diphenylmethyl]-1H-imidazole] (CAS 23593-75-1) was obtained from Sigma-Aldrich, Italy (Milan) while the degradation product (2-chlorophenyl)bisphenylmethanol (CAS 66774-02-5) was obtained from LGC Standards, Italy. Coumarin (CAS 91–64–5) was obtained from Merck, Denmark. Stock solutions were prepared in autoclaved distilled deionized water and stored in the dark at 5 °C.

2.2. Vacuum-UV irradiation (VUV/UVC)

The effect of vacuum-UV irradiation of clotrimazole in water was investigated in a continuous-flow VUV photoreactor from ULTRAAQUA A/S (Aalborg, Denmark). The VUV photoreactor consisted of a tubular stainless-steel reactor with an inner diameter of 53 mm, a length of 1270 mm, and a reactor volume of 1.7 L (Fig. S1). The photoreactor was connected to a 2.3 L stainless steel reservoir and a diaphragm pump (Siebec, pompe M7) operated at 2.0–2.2 L/min for recirculation. The reservoir was equipped with a magnetic stir bar to facilitate mixing, and a stainless-steel cooling spiral operated at 10 °C to prevent heating. The average retention time and photoreactor passages as a function of flowrate were calculated using tracer studies with Safranin O (1 mg/L).

The photoreactor was equipped with a low-pressure high output amalgam VUV Hg lamp with a 19 mm diameter, and a length of 1050 mm (UltraTherm 200 W LPHO TOC UV, ULTRAAQUA A/S, Denmark). The UV lamp simultaneously emitted UVC (254 nm) and VUV (185 nm) at a 4:1 ratio with radiation flux of 56 W and 14 W, respectively (hereafter referred to as VUV/UVC irradiation). The VUV lamp was located inside a high purity 28 mm diameter quartz tube transparent to both wavelengths. The theoretical thickness of the water film around the quartz sheath was 12.5 mm. The reactor was equipped with an UVC sensor and the maximum irradiance in drinking water was 340 W m^{-2} for UVC corresponding to 85 W m^{-2} of VUV.

VUV irradiation experiments were conducted by loading the photoreactor and reservoir with a total of 4 L of water spiked with clotrimazole with different nominal concentrations (1 μg/L to 4 mg/L). The water was recirculated for 64 min and 10 mL samples were collected at the outlet of the photoreactor before turning on the VUV lamp (0 min), and at 1, 2, 4, 8, 16, 32 and 64 min after turning on the UV lamp ("post VUV") corresponding to 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 J/cm^2 dose exposure.

Water sample was collected at Aalborg Municipality, Denmark. The source water in Aalborg Municipality is hard groundwater (11°dH) abstracted directly from chalk aquifers and contains 264 mg/L bicarbonate (HCO_3^-). No water treatment or disinfection is employed by the municipality before distribution of the drinking water to the consumers. The drinking water is naturally nutrient poor with a concentration of non-volatile organic carbon (NVOC), NO_3^-, NH_3/NH_4^+, NO_2^-, and total P of 0.99 mg/L, 1.3 mg/L, 0.007 mg/L, $<$ 0.001 mg/L, and 0.01 mg/L, respectively. The water temperature, pH and turbidity were 8.8 °C, 7.6 and $<$ 0.13 FTU, respectively.

2.3. UVC irradiation

The effect of UVC irradiation of clotrimazole in water was investigated to compare results from combined VUV/UVC irradiation (see above) with effect of UVC irradiation alone (no VUV). Stock solutions of clotrimazole (4 mg/L) were exposed to UVC irradiation in 10 mm 3.5 mL quartz cuvettes (Science Outlet Optical Quartz QS10 and Hellma Precision Quartz Suprasil QS10) using an 8 W UVP 3UV lamp (Analytic Jena, USA). The UVC irradiation was carried out in a smaller volume compared to the VUV/UVC irradiation because a monochromatic UVC lamp was not available for the large VUV reactor system. UVC Irradiation intensity was measured using an Extech SDL470 Light meter equipped with an UVC sensor. The UVC irradiation intensity at 10 cm distance from the UVP 3UV lamp was 60 μW/cm^2/sec. UVC doses (J/cm^2) were calculated from the measured UV irradiation intensity (μW/cm^2/sec) and the exposure time (sec). The UV exposure doses were varied by changing the exposure times.

2.4. Detection of active oxygen species

The formation of H_2O_2 in aqueous samples after VUV/UVC treatment

was assessed by spectrophotometry using the vanadate method in which a peroxo complex is measured as absorbance at 450 nm [31]. The formation of •OH in aqueous samples during VUV/UVC irradiation was investigated by the addition of coumarin (1 mM) as a probe molecule. The fluorescence resulting from the reaction between hydroxyl radicals and coumarin to form the highly fluorescent 7-hydroxycoumarin was measured over time using a Victor X2 Multilabel Plate Reader (Perkin Elmer).

2.5. Clotrimazole degradation and occurrence of transformation products

The concentration of clotrimazole over time was measured by HPLC with UV detection at 230 nm (Summit - Dionex Corporation). The HPLC was equipped with a Luna 5 μ C18 100 Å (250 × 4.60 mm) column and acetonitrile/water (50:50 v:v) was used as mobile phase at a flow of 1 mL/min.

Non-target liquid chromatography-high resolution mass spectrometry analysis (LC-HRMS) of water samples with clotrimazole was performed before and after UV exposure to identify transformation products. Analyses were carried out using an Ultimate 3000 High-Pressure Liquid Chromatography coupled through an ESI source to an LTQ-Orbitrap mass spectrometer (Thermo Scientific). Chromatographic separation was achieved using a reversed-phase C18 column (Phenomenex Luna, 150 × 2 mm, 3 μm, 110 Å; Phenomenex, Italy) by injecting a 10 μL sample volume with a mobile phase consisted of a mixture of 0.1 mM formic acid (eluent A) and acetonitrile (eluent B). The gradient profile started with 5% B, increased up to 100% B in 40 min and to 100% A in 10 min. Samples were ionized in both positive and negative ionization modes. The LC effluent was delivered to the ESI ion source using N_2 as sheath and auxiliary gas with the following parameters: sheath gas 34 arbitrary unit (arb), auxiliary gas 15 arb, capillary voltage 4.48 kV, and capillary temperature of 270 °C. Full mass spectra were acquired in the positive ion mode with a resolution of 30.000. Data analysis was performed using the MZmine 2.53 for peak alignment, peak grouping, background noise and retention time correction, and the METLIN database was used to identify the transformation products.

Samples at initial concentration of 1 μg/L were preconcentrated before analysis using a freeze-dryer (LABOGENE – CoolSafe 55–110). After drying 15 mL of the sample, the reconstituted with 500 μL of acetonitrile (final pre- concentration factor of 30) for the LC-HRMS analysis.

Total organic carbon (TOC) was measured using a Shimadzu TOC-5000 analyzer with catalytic oxidation on Pt at 680 °C. The calibration was performed using standards of potassium phthalate.

2.6. Toxicity test with the luminescent bacterium Aliivibrio fischeri

Toxicity screening of samples with clotrimazole and (2-chlorophenyl)bisphenylmethanol were examined in a standard inhibition tests with the luminescent bacterium *Aliivibrio fischeri* (*ISO 11348–1*, 2007) [32]. *A. fischeri* DSM 7151 was incubated in white 96-well microplates (CulturPlate, Perkin Elmer) with serial 2-fold dilutions of clotrimazole and (2-chlorophenyl)bisphenylmethanol resulting in 10 different nominal concentrations and 4 replicates in each dilution series. Changes in bioluminescence was quantified after 30 min using a Victor X2 Multilabel Plate Reader (Perkin Elmer). The toxicity to *A. fischeri* was examined before and after exposure of aqueous solutions of clotrimazole and (2-chlorophenyl)bisphenylmethanol to different UV irradiation regimes.

2.7. Toxicity test with the luminescent yeast Saccharomyces cerevisiae BLYR

The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to a single-celled yeast was examined in an inhibition test with the bioluminescent yeast reporter strain *Saccharomyces cerevisiae* BLYR (490 Biotech, USA). This yeast strain produces light continuously by constitutive expression of the luxCDABE genes from *Photorhabdus luminescens* and the frp gene from *Vibrio harveyi* [33]. We used this engineered yeast to develop a bioassay in which the endpoint was inhibition of luminescence after growth for 72 h.

S. cerevisiae BLYR was grown for 24–48 h at 25 °C in YMM medium [34] until luminescence was detectable. The culture was then diluted 1:100 in YMM medium and used as inoculum in the bioassays. 2-fold dilutions of clotrimazole were prepared in 96-well white microplates (CulturPlate, Perkin Elmer) by serially diluting 150 μL of an aqueous solution with the chemical in 150 μL YMM medium. After dilution of clotrimazole, 150 μL of 1:100 diluted *S. cerevisiae* BLYR culture was added to each well resulting in a final liquid volume of 300 μL in each well. Sealed plates were incubated with shaking for 72 h ± 2 h at 25 °C. Changes in bioluminescence was then quantified using a Victor X2 Multilabel Plate Reader (Perkin Elmer). The bioassay with *S. cerevisiae* BLYR included four replicates of blanks (medium only), controls (no clotrimazole), and each of the 10 clotrimazole concentrations. The toxicity of clotrimazole to *S. cerevisiae* BLYR was examined before and after exposure of aqueous solutions of the chemical to different UV irradiation regimes.

2.8. Toxicity test with the fungus Fusarium graminearum

The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to a filamentous fungus was examined in an inhibition test with the plant pathogen *Fusarium graminearum.* We used this fungal strain to develop a Fusarium Toxicity assay (FUTOX) in which the endpoint was inhibition of enzyme activity after growth for 72 h. *F. graminearum* was cultivated at 25 °C in a Fusarium Minimal Medium (FMM) with the following composition (g/L): 0.12 Na_2SO_4; 0.05 $MgSO_4*7H_2O$; 0.008 $CaCl_2*2H_2O$; 0.268 NH_4Cl; 5.0 KNO_3; 1.14 Na_2HPO_2; 0.272 KH_2PO_4; 0.5 Yeast extract; 1.0 Proteose peptone; 10.0 Glucose; 10.0 Maltose. The medium was supplemented with the following trace elements (mg/L): 1.39 $FeSO_4*7H_2O$; 0.054 $ZnCl_2$; 0.068 $CuCl_2*2H_2O$; 0.021 NaBr; 0.024 $Na_2MoO_2*2H_2O$; 0.079 $MnCl_2*4H_2O$; 0.033 KI; 0.025 H_3Bo_3; 0.048 $CoCl_2*6H_2O$; 0.048 $NiCl_2*6H_2O$.

2-fold dilutions of clotrimazole and (2-chlorophenyl)bisphenylmethanol were prepared in 96-well black microplates (CulturPlate, Perkin Elmer) by serially diluting 150 μL of an aqueous solution with the chemical in 150 μL FMM medium. After diluting the chemical, 150 μL of diluted *F. graminearum* culture was added to each well resulting in a final liquid volume of 300 μL in each well. The *F. graminearum* culture used as inoculum in the FUTOX assay consisted of a 72–96 h old culture in FMM diluted to A600 = 0.01 in FMM.

Sealed plates were incubated with shaking for 72 h ± 2 h at 25 °C. Activity of chitinase (1→4)-2-acetamido-2-deoxy-β-D-glucan glycanohydrolase) in *F. graminearum* was measured by adding 30 μL of the fluorescent substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-Methylumbelliferyl 2-Acetamido-2-deoxy-β-D-glucopyranoside). The fluorescent chitinase substrate was added to each well from a concentrated stock solution in dimethyl sulfoxide to obtain a final concentration of 10 μM. After 60 min and 120 min incubation at 25 °C, fluorescence was quantified in each well using a Victor X2 Multilabel Plate Reader with a 355 nm excitation and 460 nm emission filter (Perkin Elmer). The bioassay with *F. graminearum* included eight replicates of blanks (medium only), controls (no compound), and each of the 10 pollutant concentrations. The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to *F. graminearum* was examined before and after exposure of aqueous solutions of the chemicals to different UV irradiation regimes.

2.9. Toxicity test with the green microalga Raphidocelis subcapitata

The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to phytoplankton was examined in an inhibition tests with the

unicellular green microalgae *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* [35]). *R. subcapitata* (MicroBioTests Inc.) was cultivated in alga test medium at 23 ± 2 °C and continuous illumination at 6500 lux (ISO 8692, 2012 [35]).

2-fold dilutions of clotrimazole and (2-chlorophenyl)bisphenylmethanol were prepared in 96-well clear Nunclon microplates (Thermo Scientific) by serially diluting 150 µL of an aqueous solution with the chemical in 150 µL alga test medium. After dilution, 150 µL of diluted *R. subcapitata* culture (1:50) was added to each well resulting in a final liquid volume of 300 µL in each well. Plates were incubated for 72 h at 23 °C on a shaker at 70 rpm with continuous illumination (6500 lux). Growth was measured after 0, 24 h, 48 h and 72 h as absorbance at 450 nm using a Thermo Multiskan Plate Reader (Thermo Scientific). The bioassay with *R. subcapitata* included eight replicates of blanks (medium only), controls (no compounds), and each of the 10 pollutants concentrations. The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to *R. subcapitata* was examined before and after exposure of aqueous solutions of the chemicals to different UV irradiation regimes.

2.10. Toxicity test with the crustacean Daphnia magna

The toxicity of clotrimazole and (2-chlorophenyl)bisphenylmethanol to zooplankton was examined in inhibition tests with the crustacean *D. magna* (ISO 6341, 2012). The toxicological endpoint was inhibition of mobility determined by visual inspection of the animals [36]. *D. magna* STRAUS was cultivated from a laboratory clone originating from pure culture ephippia. Each treatment consisted of 20 juvenile animals distributed among 4 glass vials with 5 animals and 20 mL freshwater medium in each 30 mL vial. The mobility of each animal was determined after 24 h and 48 h (ISO 6341, 2012) [36].

2.11. Data analysis and statistics

The toxic response measured for all endpoints were expressed as inhibition (I) relative to control samples: $I = 1 - (R_i / R_c)$, where R_i and R_c are responses measured for inhibited and control samples, respectively. Control samples included water samples with UV exposure but without clotrimazole or (2-chlorophenyl)bisphenylmethanol to assess any toxicity associated with active oxygen species generated during irradiation.

Concentration-response curves were fitted to a log-logistic model using iterative non-linear regression:

$$Response = A1 + \frac{A2 - A1}{1 + 10^{(LogEC50 - C)p}} \quad (1)$$

where A1 is the bottom asymptote, A2 is the top asymptote, C is the toxicant concentration (mg/L), EC50 is the median effective concentration (mg/L), and p is a model parameter representing the slope of the curve. Iterative non-linear regressions and calculation of 95% confidence limits for EC50 values were performed using Prism 8.0.1 (Graphpad Software) or Origin 2019b (9.65, OriginLab Software).

3. Results

3.1. VUV/UVC and UVC degradation of clotrimazole

Clotrimazole removal during simultaneous VUV and UVC irradiation (VUV/UVC) was investigated over time and UV dose (Fig. 1). A fast removal was observed within the initial 8 min of VUV/UVC irradiation for both 4 mg/L and 1 µg/L, and the degradation appeared to follow first order kinetics (exponential decrease), with a half-life of less than 1 min (0.78 J/cm^2) for both concentrations. About 95% drug removal was observed after 32 min for 4 mg/L and 97% removal for 1 µg/L at the same irradiation dose (25 J/cm^2). Hence, a faster drug removal was observed at trace levels (1 µg/L) compared to 4 mg/L with a $k = 0.09$ and $k = 0.07$ min^{-1}, respectively (Fig. 1). Similarly, the TOC analysis showed a fast initial removal with 42% degradation after 4 min, and subsequent slower removal with time resulting in 92% degradation after 64 min (Fig. 1). The TOC fraction included the added clotrimazole but also natural organic matter in the water. Generation of hydroxyl radicals in the water appeared to be slightly greater in the initial phase of the VUV/UVC irradiation (Fig. S5).

The effect of monochromatic UVC (254 nm) on clotrimazole removal was also investigated, as shown in Fig. 1b. Exposure of 4 mg/L clotrimazole to 5 J/cm^2 removed more than 75% of the drug while 40 J/cm^2 resulted in > 99% removal.

3.2. Identification of clotrimazole transformation products after UVC and VUV/UVC irradiation

Samples were analysed by liquid chromatography high-resolution mass spectrometry (LC-HRMS) to identify aqueous clotrimazole transformation products after exposure to VUV/UVC and UVC irradiation. As summarized in Table 1, a total of 7 transformation products of clotrimazole ($C_{22}H_{17}ClN_2$) were identified after VUV/UVC treatment, resulting manly from the hydroxyl group addition to the clotrimazole molecule and/or loss of the imidazole group. In detail, 3 isomers of the

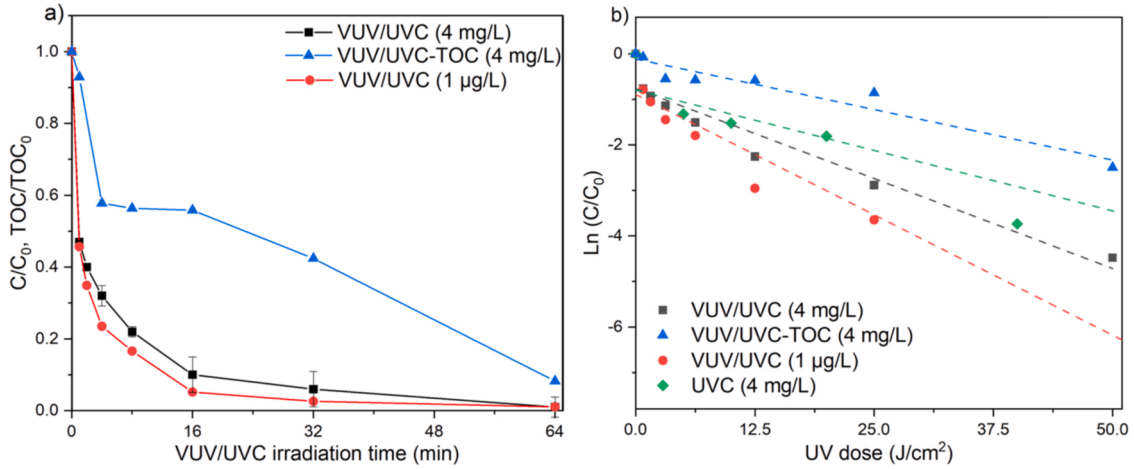


Fig. 1. Clotrimazole degradation (C_0 = 4 mg/L and C_0 = 1 µg/L) and TOC removal (C_0 = 4 mg/L) as a function of VUV/UVC irradiation time (Panel a). Ln(C/C$_0$) values for clotrimazole and TOC removal as function of UV dose for VUV/UVC irradiation and monochromatic UVC (C_0 = 4 mg/L) (Panel b).

Table 1
Transformation products identified by LC-HRMS analysis after VUV/UVC and UVC irradiation of aqueous clotrimazole. [a])not observed after VUV/UVC irradiation; [b])not observed after UVC irradiation.

Compound	r.t. (min)	Empirical formula	m/z $[M+H]^+$	△ (ppm)
Clotrimazole	14.6	$C_{22}H_{17}ClN_2$	345.1158	1.61
TP1	21.9	$C_{19}H_{15}ClO$	295.0779	-0.02
TP2	10.9	$C_{22}H_{17}ClN_2O$	361.1109	-1.57
TP3	13.7			
TP4	14.3			
TP5[a])	15.4			
TP6	14.4	$C_{20}H_{17}ClN_2$	321.1160	0.70
TP7[b])	13.4	$C_{13}H_9ClO$	217.0415	-0.55
TP8	5.1	$C_3H_4N_2$	69.0442	0.56

monohydroxylated drug on the phenyl ring were detected (TP2, TP3, TP4: $C_{22}H_{17}ClN_2O$). The hydroxylation of the imidazole ring led to the subsequent ring opening (TP6: $C_{20}H_{17}ClN_2$), while the loss of the imidazole group was followed by the hydroxylation in the tertiary carbon (TP1: $C_{19}H_{13}ClO$). The last one was confirmed by the commercial standard, Fig. S2. Additionally, further oxidation resulted in loss of one phenyl ring (TP7: $C_{13}H_9ClO$). From the profile over time of the identified photoproducts (Fig. S3), a maximum relative abundance of TPs (TP1, TP2, TP3, TP4 and TP6) was observed at 6.25 J/cm^2 (8 min) of VUV/UVC irradiation followed by a disappearance with extended exposure time and UV dose likely due to e.g., further degradation and/or mineralization (Fig. S3). Under monochromatic UVC irradiation, a similar degradation pathway was observed with formation of also a total of 7 photoproducts. However, one more isomer resulting from the drug monohydroxylation was formed after UVC (TP5) whereas the product TP7 detected after VUV/UVC was not observed under UVC (Table 1).

3.3. Selection of test organisms

Initial experiments were conducted to identify test organisms responsive to clotrimazole exposure (Fig. 2). Clotrimazole showed no effect on the traditional test organism *A. fischeri* at the investigated concentration range (0.004 – 2.0 mg/L), and it was not possible to estimate a median effective concentration (EC50). On the contrary, inhibition was observed for the yeast *S. cerevisiae BLYR*, the microalgae *R. subcapitata*, the crustacean *D. magna* and the fungus *F. graminearum* with EC50 values of 0.39 mg/L, 0.15 mg/L, 0.04 mg/L and 0.01 mg/L, respectively. Considering that the greatest inhibition was observed for *R. subcapitata*, *D. magna* and *F. graminearum,* these test organisms were selected for subsequent experiments in which changes in ecotoxicity was examined after UV treatment of aqueous clotrimazole.

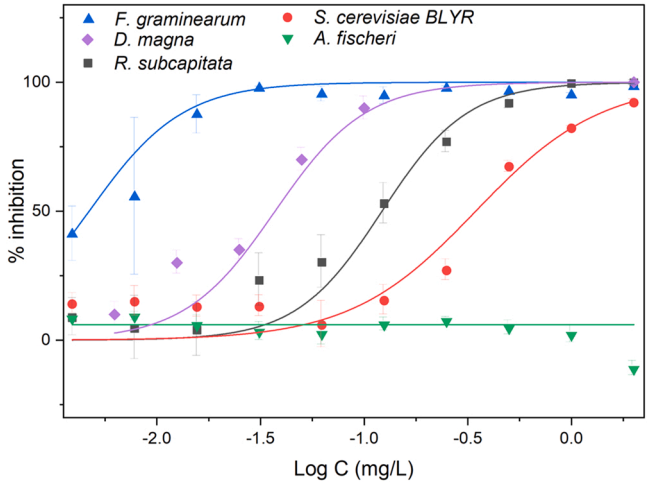


Fig. 2. Toxicity of clotrimazole to different test organisms measured as concentration-response curves. Data points represent mean ± standard error.

3.4. Toxicity of the clotrimazole transformation product (2-chlorophenyl) bisphenylmethanol

(2-chlorophenyl)bisphenylmethanol was identified as a transformation product during degradation of clotrimazole under UVC and VUV/UVC (Table 2). The product resulted from the loss of the imidazole moiety followed by the hydroxylation into the tertiary carbon. To investigate the toxicity of this clotrimazole transformation products, test organisms were exposed to TP1 in the form of the commercial standard (2-chlorophenyl)bisphenylmethanol (Fig. 3). As previously observed for clotrimazole, TP1 showed no effect on *A. fischeri* at the investigated concentration range (0.004–2.0 mg/L), but also the green microalgae *R. subcapitata* was not inhibited by this photoproduct (Fig. 3). On the contrary, inhibitory effects were observed for the yeast *S. cerevisiae BLYR,* the crustacean *D. magna* and the fungus *F. graminearum* with EC50 of 0.44 mg/L, 0.25 mg/L, and 1.90 mg/L, respectively. However, it should be noted that TP1 was much less toxic compared to the parent compound clotrimazole with higher EC50 values for the test organisms. Especially the fungus *F. graminearum* was less susceptible to TP1 and this compound appeared 190 times less toxic than clotrimazole as indicated by a much greater EC50 value.

3.5. Effect of VUV/UVC and UVC irradiation on clotrimazole toxicity

The effect of VUV/UVC and monochromatic UVC irradiation on clotrimazole toxicity was investigated using *R. subcapitata*, *D. magna* and *F. graminearum* as test organisms as shown in Fig. 4 and Fig. 5. To investigate the impact of the drug disappearance as well as the formation of transformation products towards the test organisms after VUV/UVC, samples collected before irradiation (0 J/cm^2) were compared with samples irradiated with 6.25 J/cm^2 where several TPs were present at high relative abundance (see Section 3.2), and samples irradiated with 25 J/cm^2 which was the dose resulting in 95% drug removal (see Section 3.1).

Exposure of aqueous clotrimazole to different UV doses had a substantial impact on the toxicity to the test organisms as shown in Fig. 4 and Table 2. Changes in growth of *R. subcapitata* after VUV/UVC irradiation demonstrated a noticeable decrease in aqueous clotrimazole toxicity after 6.25 J/cm^2 leading to a 6-fold increase of the EC50 value from 0.29 mg/L to 1.63 mg/L. Furthermore, 25 J/cm^2 VUV/UVC irradiation led to an additional decrease in clotrimazole toxicity with no or low observed inhibitory effect on the growth of the green microalga at nominal concentrations up to 2.0 mg/L (Fig. 4a). As a result of the low inhibition after VUV/UVC, it was no longer possible to estimate an EC50 value (Table 2). Similarly, clear decreases in inhibitory effects of clotrimazole to the fungus *F. graminearum* was observed after VUV/UVC irradiation with 6.25 J/cm^2 and 25 J/cm^2, causing a fivefold (EC = 0.09 mg/L) and 47-fold (EC = 0.94 mg/L) decrease in aqueous clotrimazole toxicity, respectively (Fig. 4b and Table 2). For the crustacean *D. magna,* sample exposed to 6.25 J/cm^2 showed a 31-fold decrease in toxicity with EC50 values increasing from to 0.03 mg/L to 0.94 mg/L (Fig. 4c and Table 2). Interestingly, no inhibitory effect of aqueous

Table 2
Median effective concentration (EC50) for the test organisms before and after exposure of clotrimazole to VUV/UVC and UVC at different doses. N.D.: not determined.

Test organism	EC50 (mg/L)				
	Before UV	VUV/UVC dose		UVC dose	
		6.25 J/cm^2	25 J/cm^2	5 J/cm^2	40 J/cm^2
R. subcapitata	0.29	1.63	> 2.0	0.19	0.66
F. graminearum	0.02	0.09	0.94	0.25	1.23
D. magna	0.03	0.94	> 2.0	N.D.	N.D.

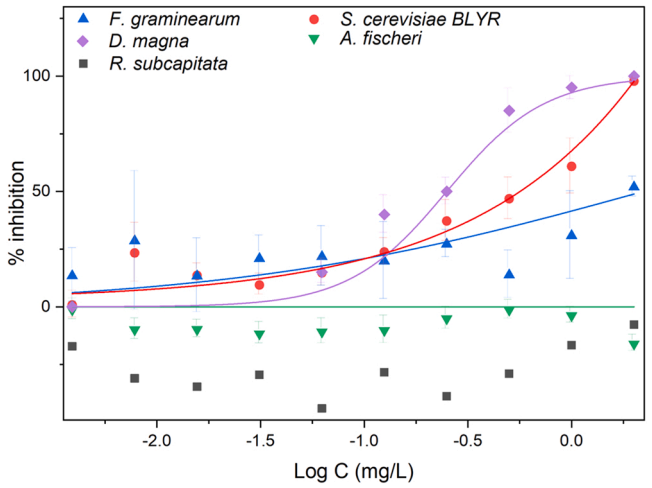


Fig. 3. Toxicity of (2-chlorophenyl)bisphenylmethanol (TP1) to different test organisms measured as concentration-response curves. Data points represent mean ± standard error.

clotrimazole to *D. magna* could be detected after VUV irradiation with 25 J/cm^2 (Fig. 4c). To further illustrate the relationship between VUV/UVC treatment and decreases in toxicity, Fig. 4d shows how different UV doses promoted an attenuation of toxicity (1/EC50) of aqueous clotrimazole to all the test organisms.

The effects of VUV/UVC irradiation on toxicity of aqueous clotrimazole was compared with the effects of monochromatic UVC irradiation (Fig. 5). UVC irradiations led to a decrease in clotrimazole toxicity to *R. subcapitata* and *F. graminearum* as shown in Fig. 5. As shown in Fig. 1, an irradiation with 40 J/cm^2 UVC resulted in almost complete clotrimazole removal, and this treatment subsequently decreased the apparent toxicity twofold to the green microalgae *R. subcapitata* as indicated by a change in EC50 from 0.29 mg/L to 0.66 mg/L (Fig. 5a and Table 2). Similarly, 40 J/cm^2 UVC decreased the growth inhibitory effect of the drug to *F. graminearum* (Fig. 5b), and for this test organism the EC50 increased 62-fold (Table 2).

4. Discussion

UV based AOPs allow the removal of micropollutants by both, direct and indirect photolysis [37,38]. Direct photolysis involves light absorption by the pollutant with subsequent transformation, while during indirect photolysis the degradation is mediated by generated reactive species. The use of the VUV processes with radiation bellow 200 nm promotes the photolysis of water allowing the in situ generation of reactive oxygen species such as hydroxyl radical ($^{\bullet}$OH), superoxide anion ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) [24,26,27], that can lead the indirect photolysis of organic pollutants. Moreover, the VUV lamps used in the present study are characterized by combined radiation at 185 nm and 254 nm (UVC), and the latter wavelength can also contribute to pollutant degradation by direct photolysis if absorbed by the molecule. In addition, UVC has a greater transmission in water compared to VUV at 185 nm which can facilitate chemical reactions further from the lamp surface.

VUV/UVC irradiation (combined 185 nm and 254 nm) showed high efficiency removing aqueous clotrimazole with more than 50% abatement in just one 1 min of exposure (0.78 J/cm^2) followed by a slower removal with increasing treatment in accordance with first order rection kinetics (Fig. 1). Clotrimazole degradation during UV photolysis investigated by LC-HRMS pointed to a pathway that resulted mainly from

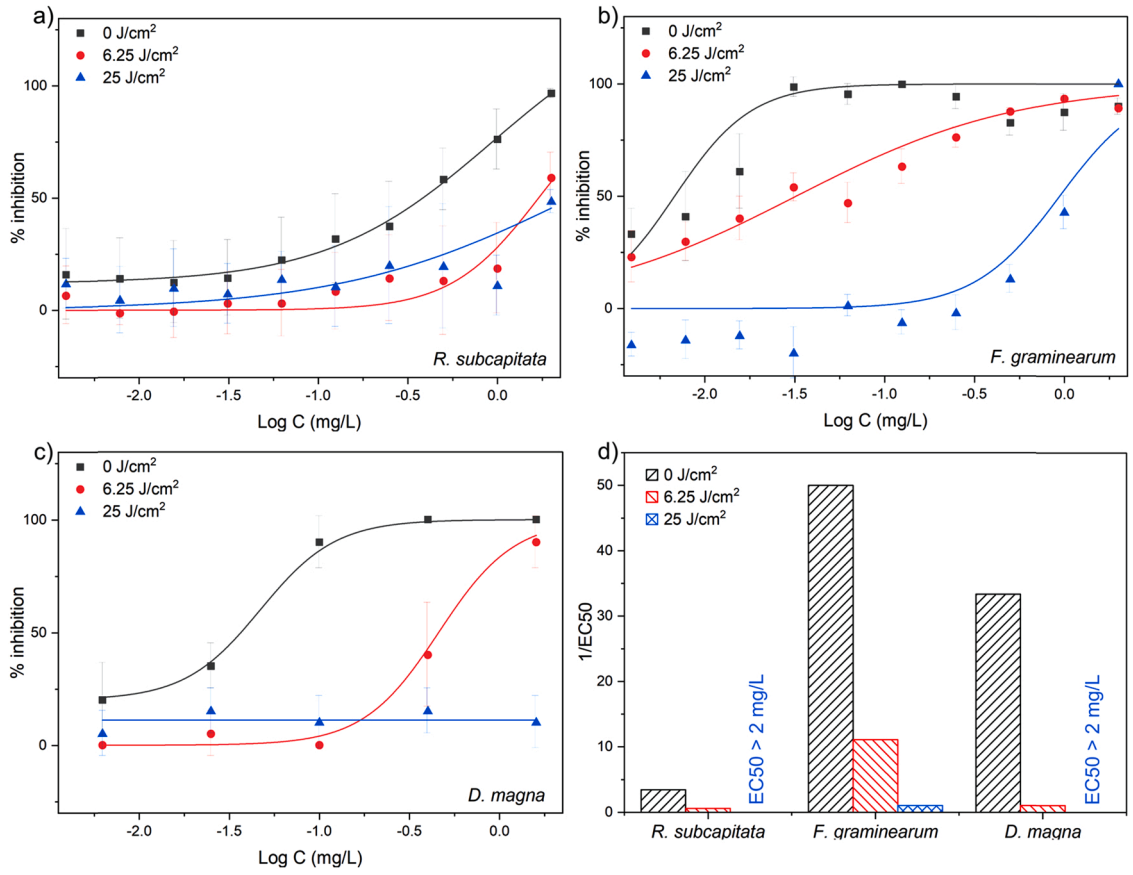


Fig. 4. Effect of VUV/UVC irradiation of aqueous clotrimazole on the toxicity to *R. subcapitata* (a), *F. graminearum* (b) and *D. magna* (c). Data points represent mean ± standard error. Panel d) shows the 1/EC50 values for the test organisms as a function of the VUV/UVC dose.

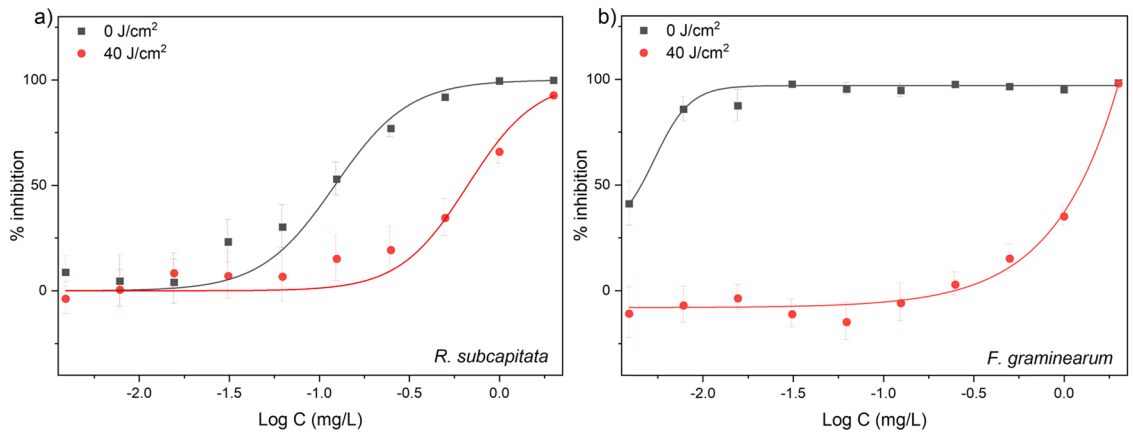


Fig. 5. Effect of monochromatic UVC irradiation of aqueous clotrimazole on the toxicity to *R. subcapitata* (a) and *F. graminearum* (b). Data points represent mean ± standard error.

hydroxyl group addition in the phenyl ring and/or imidazole moiety with further oxidation reactions resulting in the loss of the phenyl ring (Scheme 1). Clotrimazole has an unsubstituted imidazole moiety, which is probably why the ring opening or loss of imidazole moiety was the main route of drug degradation leading to the formation of (2-chlorophenyl)bisphenylmethanol (TP1), as previously reported for clotrimazole stability studies in the presence of photocatalysts [20]. The observed TPs matched with those previously described during the photocatalytic degradation using semiconductors catalysts under UVA irradiation [20]. However, the higher number of TPs reported in the photocatalytic system resulted mainly from the drug multihydroxylation and can be explained by the higher ability to generate hydroxyl radicals relatively to the UV based AOPs, yet the latter does not require the addition of chemicals or catalysts. The formation of hydroxyl radicals in the present study was substantiated by the occurrence of the highly fluorescent 7-hydroxycoumarin after VUV/UVC irradiation of water spiked with coumarin (Fig. S5). The reaction between coumarin and hydroxyl radicals supported the involvement of $^{\bullet}OH$ in the degradation processes as previously reported for VUV irradiation [26,39].

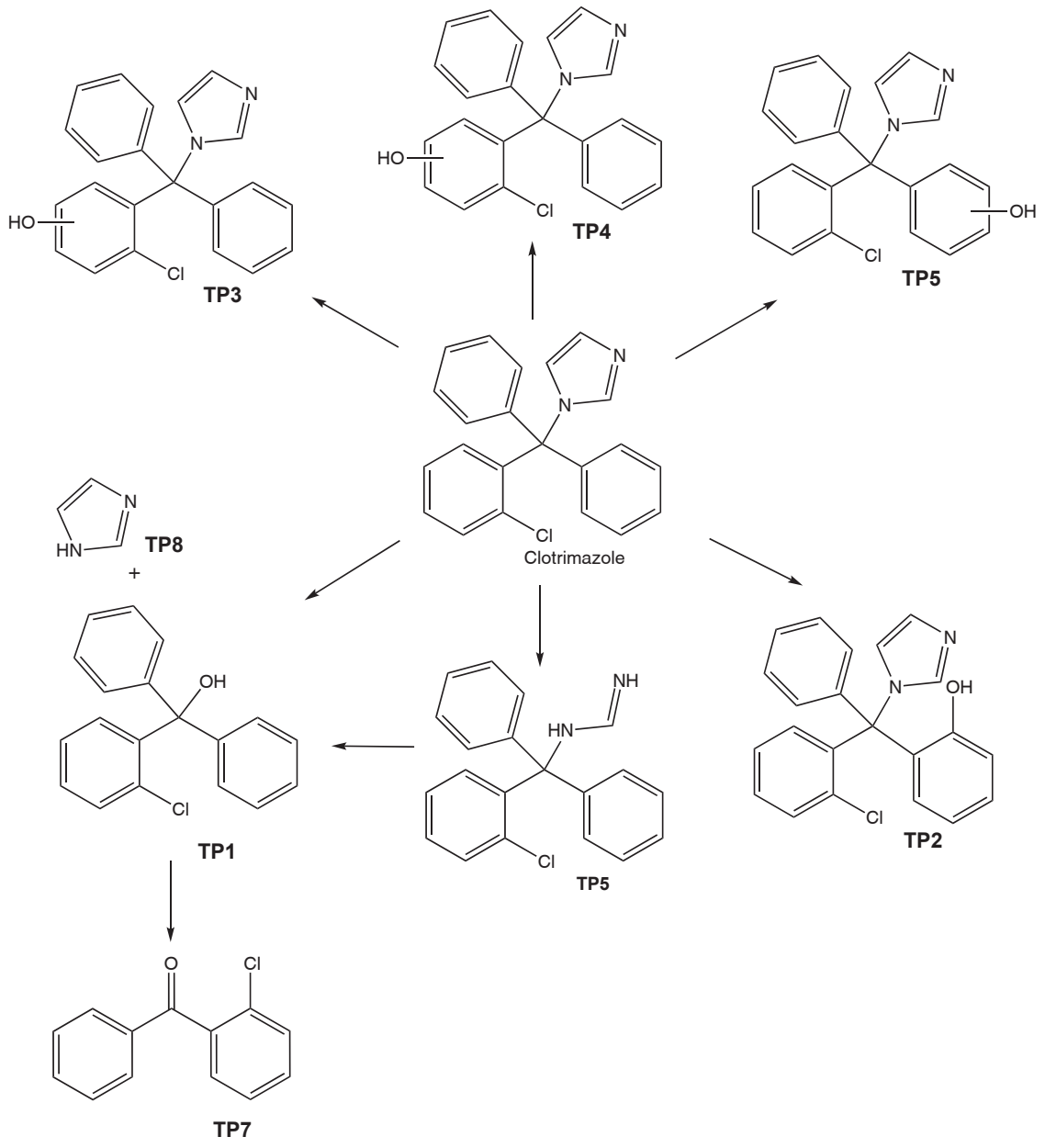
H_2O_2 is one of the products formed during VUV/UVC treatment of water [26]. In our study, we detected H_2O_2 during VUV/UVC irradiation of water in the absence of clotrimazole and the concentration increased with increasing irradiation time up to 19.7 μM after 64 min (Fig. S4). H_2O_2 is generally toxic to many organisms and should be considered carefully when applying AOP technology. Fortunately, the trace levels of H_2O_2 observed in the present study did not show any detectable inhibitory effects towards the test organisms (data not shown). This is in line with guidelines in many countries where maximum acceptable concentration of H_2O_2 in drinking water is often at the ppm level which is above the concentrations detected in the current study. Furthermore, H_2O_2 concentrations may be controlled during VUV/UVC treatment (if needed) by applying shorter irradiation time and UV doses (Fig. S4).

Organisms from different trophic levels have been reported to be adversely impacted by the clotrimazole molecule [1,5,9]. However, to the best of our knowledge the environmental fate of clotrimazole including occurrence of degradation products is still not known. Due to substantial lipophilicity and environmental persistence, clotrimazole is frequently detected in WWTPs sewage sludge used for soil amendment to promote agricultural crops [1,40]. Sabourin et al. [41] investigated the antifungal fate in agricultural soil and reported the identification of only (2-chlorophenyl)bisphenylmethanol as a degradation product. Moreover, this compound is of particular interest because it can also reach the environment resulted from the drug metabolization once it is reported as a metabolite observed in human urine and faeces [42,43]. In the present study, (2-chlorophenyl)bisphenylmethanol (TP1) was observed as a major transient transformation product after VUV/UVC treatment of water containing clotrimazole. The relatively low inhibitory effect of this photoproduct to our test organisms suggested less toxic properties compared to the clotrimazole parent molecule (Fig. 3). The inferior effect can be attributed to the absence of the imidazole moiety which is likely the active functional group, in relation to fungi, algae and crustaceans [9,44].

Our study demonstrated that VUV/UVC irradiation of clotrimazole in water can result in a decrease in overall toxicity of aqueous samples to a range of test organism. The substantial decrease in the inhibition of the green microalgae *R. subcapitata*, the crustacean *D. magna* and the fungus *F. graminearum* after exposure to 25 J/cm^2 VUV/UVC can be explained by the 95% drug removal and generation of apparently less toxic transformation products (Fig. 4). Similarly, the observed degradation profile observed under monochromatic UVC irradiation can be explained mainly by the drug direct photolysis due to their absorbance at 254 nm [45]. The almost complete drug removal observed after exposing aqueous clotrimazole to 40 J/cm^2 UVC support the decrease in toxicity towards *R. subcapitata* and *F. graminearum*. Comparable decreases in ecotoxicity after UVC irradiation has recently been observed for the herbicide glyphosate where UV doses of 20 J/cm^2 resulted in a 2–23 times decrease in toxicity to different test organisms [46]. Hence, VUV/UVC and UVC treatment generally appeared to generate less bioactive transformation products with lower overall toxicity than the parent compounds. Such products may subsequently be biodegraded in the environment or perhaps mineralized by further VUV/UVC treatment. Collectively, the results of the present study support studies suggesting a potential for use of VUV/UVC based technology to treat and detoxify water containing organic pollutants such as pharmaceutical and personal care products [27,47,48].

5. Conclusions

Our results suggest that non-invasive VUV/UVC can efficiently remove the persistent pollutant clotrimazole from water via direct and indirect photolysis and consequently decrease the overall toxicity through the formation of less toxic intermediates. A battery of test organisms that included *Aliivibrio fischeri, Raphidocelis subcapitata, Saccharomyces cerevisiae, Fusarium graminearum* and *Daphnia magna* were applied to assess decreases in toxicity after VUV/UVC irradiation. Test organisms from different trophic levels were included to better assess biological effects of all bioactive compounds in VUV/UVC treated samples including transformation products. LC-HRMS analysis identified a total of 8 transformation products resulting from VUV/UVC degradation of clotrimazole. VUV/UVC treatment efficiently removed the antifungal agent even at trace levels offering a potentially attractive solution for water remediation without the addition of supplementary



Scheme 1. Proposed degradation pathways for clotrimazole during VUV/UVC and UVC irradiation.

oxidants or catalysts.

CRediT authorship contribution statement

Nuno P.F. Gonçalves: Investigation, Writing – original draft, Writing – review & editing, Conceptualization. **Oihane del Puerto**: Investigation, Writing – review & editing, Formal analysis. **Claudio Medana**: Methodology, Formal analysis. **Paola Calza**: Conceptualization, Writing – review & editing, Funding acquisition. **Peter Roslev**: Conceptualization, Writing – review & editing, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.106275.

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